

PCT/IB05 / 00382

( 14.02.05 )

PA 1251300

REC'D 14 FEB 2005

WIPG

POT

# THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

November 23, 2004

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: 60/548,719

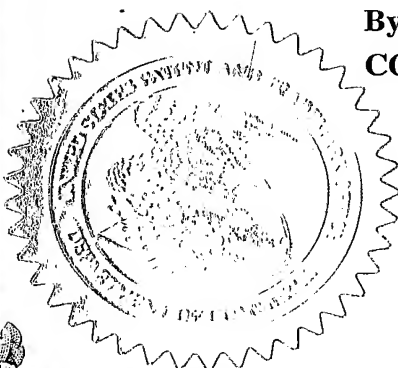
FILING DATE: February 27, 2004

PCT/IB05/00382

## PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN  
COMPLIANCE WITH RULE 17.1(a) OR (b)

By Authority of the  
COMMISSIONER OF PATENTS AND TRADEMARKS



  
M. K. HAWKINS  
Certifying Officer

16805 U.S. PTO  
022704

PTO/SB/16(08-03)

Approved for use through 7/31/2003. OMB 0651-0032  
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.  
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

**PROVISIONAL APPLICATION FOR PATENT COVER SHEET**

**This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).**

**Express Mail Label No. EV 268119131 US**

**INVENTOR(S)**

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Hiroko	Kachi	Taketoyo-cho, Chita-gun, Aichi-ken, Japan
Katsuhiro	Shinjo	Taketoyo-cho, Chita-gun, Aichi-ken, Japan
Yasuhito	Taniguchi	Taketoyo-cho, Chita-gun, Aichi-ken, Japan
Kimihiko	Tomotoshi	Taketoyo-cho, Chita-gun, Aichi-ken, Japan

**GPR35**

Direct all correspondence to:

**CORRESPONDENCE ADDRESS**

☒ Customer Number **28523**

☐ Firm or Individual Name

Address

Address

City

State

Zip

Country

Telephone

Fax

**ENCLOSED APPLICATION PARTS (check all that apply)**

<input checked="" type="checkbox"/> Specification	Number of Pages	36	<input checked="" type="checkbox"/> Claims	5
<input checked="" type="checkbox"/> Drawing(s)	Number of Sheets	10	<input checked="" type="checkbox"/> Abstract	1
<input checked="" type="checkbox"/> Application Data Sheet. See 37 CFR 1.76		2	<input checked="" type="checkbox"/> Sequence Listing	9

**METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT**

<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27	<b>FILING FEE AMOUNT(\$)</b>
<input type="checkbox"/> A check or money order is enclosed to cover the filing fees	<b>\$160.00</b>
<input checked="" type="checkbox"/> The Director is hereby authorized to charge all required filing fees to, and credit any overpayment to Deposit Account Number: <b>16-1445</b>	
<input type="checkbox"/> Payment by credit card Form PTO-2038 is attached.	

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

SIGNATURE

TYPED or PRINTED NAME

*John A. Wichtowski*  
John A. Wichtowski

DATE:

REGISTRATION NO

(if appropriate)

Docket Number.

**27 FEBRUARY 2004**

48,032

PC32099

**USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT  
ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT**

This collection of information is required by 37 CFR 1.51. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Mail Stop Provisional Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.  
If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

### Application Information

Application Type:: Provisional  
Subject Matter:: Utility  
Title:: GPR35  
Attorney Docket Number:: PC32099  
CD-ROM or CD-R?:  
Number of CD disks:  
Number of copies of CDs:  
Sequence submissions?: 9 pages  
Computer Readable Form (CRF?):  
Number of copies of CRF:  
Suggested Drawing Figure:: 10 pages  
Total Drawing Sheets::

### Inventor Information

Inventor Authority Type:: INVENTOR  
Primary CitizenshipCountry:: Japanese  
Given Name:: Hiroko  
Family Name:: Kachi  
City of Residence:  
State or Prov of Residence:  
Country of Residence:: Japan  
Street:: 2, Aza 5-gochi  
City:: Taketoyo-cho, Chita-gun  
State or Province:: Aichi-ken  
Postal or Zip Code:: 470-2393  
Country:: Japan  
Inventor Authority Type:: INVENTOR  
Primary CitizenshipCountry:: Japanese  
Given Name:: Katsuhiko  
Family Name:: Shinjo  
City of Residence:  
State or Prov of Residence:  
Country of Residence:: Japan  
Street:: 2, Aza 5-gochi  
City:: Taketoyo-cho, Chita-gun  
State or Province:: Aichi-ken  
Postal or Zip Code:: 470-2393  
Country:: Japan

**Application Data Sheet**  
**Express Mail No. EV 268119131 US**

Inventor Authority Type:: INVENTOR  
Primary CitizenshipCountry:: Japanese  
Given Name:: Yasuhito  
Family Name:: Taniguchi  
City of Residence::  
State or Prov of Residence::  
Country of Residence:: Japan  
Street:: 2 Aza, 5-gochi  
City:: Taketoyo-cho, Chita-gun  
State or Province:: Aichi-ken  
Postal or Zip Code:: 4702393  
Country:: Japan  
Inventor Authority Type:: INVENTOR  
Primary CitizenshipCountry:: Japanese  
Given Name:: Kimihiko  
Family Name:: Tomotoshi  
City of Residence::  
State or Prov of Residence::  
Country of Residence:: Japan  
Street:: 2, Aza 5-gochi.  
City:: Taketoyo-cho, Chita-gun  
State or Province:: Aichi-ken  
Postal or Zip Code:: 470-2393  
Country:: Japan

**Correspondence Information**

Correspondence Customer Number:: 28523

**Representative Information**

Representative Customer Number:: 28523

**Assignee Information**

Assignee Name::

## GPR35

### Technical Field of the Invention

The present invention primarily relates to function analysis of GPR35, an orphan  
5 GPCR (G protein coupled receptor), a screening method to identify modulators  
(agonists/antagonists) of the receptor, cells stably expressing the receptor, and the discovery  
of modulators of the receptor. The present invention also relates to novel rat GPR35.

### Background Art of the Invention

10 G protein coupled receptors are a large superfamily of integral membrane  
proteins, involved in a broad range of signaling pathways. Most G protein-coupled receptors  
are characterised by 7 transmembrane-spanning helices, and are therefore also called 7-  
transmembrane receptors (7TMs). There are at least several hundred members of this  
family, which include receptors responding to a wide range of different stimuli, including  
15 peptides, biogenic amines, lipids, neurotransmitters, hormones, nucleotides,  
sugar-nucleotides, cytokines, etc. Structurally, the receptors of this family consist of an  
extracellular amino terminal domain, seven membrane-spanning hydrophobic regions, six  
loop regions, three of which are extracellular, the other three being intracellular, and an  
intracellular carboxy terminal domain. The hydrophobic regions show considerable  
20 homology between the different members of this family, whereas the loop regions, as well as  
the amino and carboxy terminal domains, are quite diverse, showing high homology only  
amongst closely related receptor subtypes.

G proteins are heterotrimeric complexes, containing an  $\alpha$ , a  $\beta$ , a  $\gamma$  subunits in  
which  $\alpha$  is bigger than  $\beta$  and  $\beta$  is bigger than  $\gamma$ . At least 20 human genes are known to  
25 encode the GTP-binding  $\alpha$ -subunits. Furthermore, there are at least 5 genes encoding the  $\beta$ -  
subunits and at least 11 genes encoding  $\gamma$ -subunits.

The  $\alpha$ -subunit family is divided into four subfamilies on the basis of their  
homology:

(1) The  $G_s$  family is typically able to stimulate adenylate cyclase upon agonist binding to the  
30 receptor coupling to it, and therefore lead to an increase in intracellular cyclic AMP.

(2) The Gq family, which includes Gq $\alpha$ , G11  $\alpha$ , G14  $\alpha$ , G16  $\alpha$ , lead to activation of phospholipase C.

(3) G12 and G13 appear to regulate classes of small molecular weight G proteins and Na<sup>+</sup>-H<sup>+</sup> exchange.

- 5 (4) The Gi family typically mediate inhibition of adenylate cyclase. More than half of the  $\alpha$ -subunits are members of the Gi family and include the ubiquitously expressed and nearly identical Gi1  $\alpha$ , Gi2  $\alpha$  and Gi3  $\alpha$ , as well as several with a limited expression, such as Gz  $\alpha$ , Gox and transducin.

Initiation of signal transduction cascades involving G proteins requires the  
 10 binding of a ligand to a G protein-coupled receptor (GPCR). This results in the stabilisation of conformations of the GPCR that increases the rate of dissociation of the bound GDP from the nucleotide binding pocket of G  $\alpha$  subunit of a G protein. Binding of GTP is thus allowed. With GTP bound, the G protein dissociates into GTP-G  $\alpha$  and G $\beta\gamma$  moieties and can hence regulate directly or otherwise the activity of several enzymes, which generate intracellular  
 15 signalling molecules (e.g. cyclic AMP), or the probability of opening a range of ion channels (Gilman, A.G. (1987) Annu Rev. Biochem. 56, 614-649). Following G protein activation, hydrolysis of bound GTP by intrinsic GTPase activity terminates its ability to regulate its effectors and leads to  $\alpha$ -GDP reassociation with  $\beta\gamma$ . The G protein therefore serves a dual role, as a relay transmitting the signal from the receptor to the effector, and as a clock that  
 20 controls the duration of the signal. Therefore, depending on which GPCR couples to which G protein, very different intracellular effects can result, e.g. stimulation or inhibition of adenylate cyclase, leading to increased or decreased cyclic AMP concentrations in the cells, opening of ion channels, etc.

GPCRs are very important targets for pharmaceutical intervention. Many  
 25 agonists and antagonists of the GPCRs of known functions are important for the treatment of diseases. Examples include but are not limited to,  $\beta$ 1-adrenergic receptor antagonists used for treating hypertension, angina and heart failure, dopamine D2 antagonists used for the treatment of schizophrenia, and  $\beta$ 2-adrenergic receptor agonists used for the treatment of asthma. Over the past 15 years, nearly 350 therapeutic agents targeting GPCRs have  
 30 been introduced onto the market, and it is estimated that around 30% of clinically prescribed

drugs function as either agonists or antagonists at GPCRs.

Therefore, it is likely that amongst the orphan receptors, there are further therapeutic targets which can play a key role in treating or preventing diseases or dysfunctions, including, but not limited to, pain, cancers, obesity, eating disorders, hypertension, hypotension, heart failure, incontinence, asthma, chronic bronchitis, angina, ulcers, psychotic and neurological disorders, viral infections including HIV-1 or HIV-2, other infections, inflammatory conditions, sexual dysfunction, urogenital disorders, and many other disorders, diseases or dysfunctions. It is therefore clearly highly desirable to find the physiological function for each GPCR.

However, it is not at all easy to find the physiological function for each GPCR as discussed below.

The conserved structure and hydrophobicity profile of this receptor family has allowed the identification and cloning of some members of this family, both through cloning experiments, e.g. degenerate polymerase chain reaction or cDNA library screening with probes derived from GPCR sequences using low stringency, and through bioinformatic mining of sequence databases. For many of these receptors, it is yet unclear what their functions are. These receptors are referred to as "orphan receptors". Through detailed analysis of their sequences and comparison with the sequences of GPCRs with known ligands, hypotheses can be generated as to the class of ligands that the orphan receptor may respond to.

However, these predictions are often tenuous, and it is still difficult to identify the ligand that such an orphan receptor may respond to. This is mainly because stimulation of GPCRs can lead to a multitude of different intracellular effects, which are mediated through the coupling to different G proteins. The G proteins in turn act on a whole range of signalling proteins, including phospholipase C, adenylate cyclase and ion channels, and thereby initiate a cascade of events in the cells.

It is difficult to predict from the sequence of the GPCR which G protein it will couple to, and in some cases the resulting intracellular effects are difficult to measure. Furthermore, the usual host cells used for heterologous expression of cloned GPCRs only express certain G proteins. Therefore, if the G protein that the orphan GPCR couples to is not expressed in these cells, the binding of an agonist to this receptor will not have a

measurable effect, making it unlikely that one could find the agonist(s) for the GPCR in such a system. It is therefore necessary to engineer specialized host cell lines, expressing various heterologous G proteins, to enable the establishment of host cell systems that can lead to measurable responses for many of the different GPCRs.

5 With regard to GPR35, there are several articles that report nucleotide and/or amino acid sequence of human and/or mouse GPR35. However, none of them reveals specific physiological function of GPR35. All that has been previously disclosed merely presumes that GPR35 is a GPCR.

10 "Discovery of three novel G-protein-coupled receptor genes (O'Dowd BF et al. Genomics 47 (2), 310-313 (1998))" discloses cDNA and amino acid sequences of human GPR35. However, this report merely assumes the GPR35 is a GPCR. Needless to say, it does not mention any physiological function of the GPR35. Northern blotting was employed to determine tissue distribution. However, in rats, GPR35 expression was detected in the intestine but not in the heart, spleen, liver, lung, ovary, kidney, nor whole brain. In humans, 15 the expression was not detected in the caudate-putamen, thalamus, frontal cortex, midbrain, lung, nor adrenal. The expression in the DRG, dorsal horn, or spinal cord which is closely related to a neural activity, was therefore not detected.

"Genetic variation in the gene encoding calpain 10 is associated with type 2 diabetes mellitus (Horikawa Y et al. Nat. Genet. 26:163-175 (2000))" discloses the cloning of 20 mouse GPR35 gene. In this article, however, genes and SNPs located in the NIDDM1 region on chromosome 2 (which is suspected of relating to NIDDM), were examined at random to verify the linkage (association) between the disease and SNPs, in order to identify genetic elements related to non-insulin dependent diabetes mellitus (NIDDM) also known as type 2 diabetes. Although this article provides the findings that the GPR35 gene is located 25 in the NIDDM1 region and that there are a number of polymorphisms of human GPR35, it did not mention specific physiological functions of GPR35 nor lead to a positive conclusion concerning its relationship with NIDDM.

3 Since the sequences of human GPR35 and mouse GPR35 are known, some biotechnology companies may prepare antibodies or knockout animals according to the 30 consignment and, in general, the nondisclosure agreement. These can be employed only as

research tools, but does not clarify the functions by themselves.

The inventors of the present invention overcame these difficulties and disclosed specific physiological function of GPR35.

5

### Brief Disclosure of the Invention

The present invention encompasses the following non-limiting aspects.

One aspect of the present invention is:

a method of screening for a compound that modulates a GPR35 protein, comprising the step of

- 10 (a) contacting a GPR35 protein or a partial polypeptide thereof with a test compound.

The GPR35 protein or the partial polypeptide thereof used in the screening method of the present invention can exist in cells or a membrane fraction.

- The screening method of the present invention can be used to screen compounds that modulates neural activity. The screening method of the present invention can also be  
 15 used to screen compounds for use in the treatment or prevention of the following conditions: pain, anxiety, convulsions, cognition disorders, obesity, schizophrenia, neurodegeneration, depression, attention deficit hyperactivity disorder, mania, memory deficit, eating disorders, Parkinson's disease, Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis, drug addiction, bipolar disorders, circadian rhythm disorders, migraine, sexual dysfunctions,  
 20 sleep disorders and eating disorders.

In the screening method of the present invention, test compound can consist of a plurality of compounds. In this case, what is determined is whether these compounds can, as a combination, modulate a GPR35 protein.

- In the screening method of the present invention, a GPR protein or a partial  
 25 polypeptide thereof of the following can be used:

- (a) a polypeptide having the amino acid sequence of sequence ID. 2, 4, or 6,  
 (b) a polypeptide having the same amino acid sequence as the polypeptide of (a) except that one or more amino acids are deleted, substituted, or added,  
 (c) a polypeptide having an amino acid sequence that has at least 80% identity to the the  
 30 polypeptide of (a),

(d) a polypeptide having an amino acid sequence that has at least 90% identity to the the polypeptide of (a),

(e) a polypeptide having an amino acid sequence that has at least 98% identity to the the polypeptide of (a), and

5 (f) a human GPR35 protein, a mouse GPR35 protein, or a rat GPR35 protein.

Another aspect of the present invention is:

a method of screening for a compound that inhibits the binding of a ligand to a GPR35 protein, comprising the steps of

^(a) contacting a GPR35 protein or a partial polypeptide thereof with the ligand,

10 (b) contacting a GPR35 protein or a partial polypeptide thereof prepared in the substantially same manner as the one used in the step (a) with the ligand prepared in the substantially same manner as the one used in the step (a), in the presence of a test compound, and

(c) comparing the results of the step (a) and the step (b) to determine whether the binding of the ligand is affected by the presence of the test compound.

15 In this screening method of the binding assay, a detectable label can be bound to the ligand, and the step (c) can comprise the step of comparing the detected amount of label in the step (a) and the detected amount of label in the step (b).

In this screening method of the binding assay, the ligand can be selected from zaprinast, an analogue thereof, or a mimetic thereof.

20 Another aspect of the present invention is a screening method for agonists or antagonists of a GPR35 protein by functional assay.

In the screening method for agonists or antagonists of a GPR35 protein by functional assay, the functional response to be observed can be, for example, the increase of intracellular calcium concentration.

25 One aspect of against screening by functional assay is:

a method of screening for a compound that is an agonist of a GPR35 protein, comprising the steps of

(a) adding a test compound to cells expressing a GPR35 protein or a partial polypeptide thereof or to a membrane fraction from the cells, and

30 (b) determining whether a functional response is observed.

One aspect of against screening by functional assay is:

a method of screening for a compound that is an antagonist of GPR35 protein, comprising the steps of

(a) adding an agonist to cells expressing GPR35 protein or a partial polypeptide thereof or to  
5 a membrane fraction from the cells,

(b) adding an agonist prepared in the substantially same manner as the one used in the step  
(a) and a test compound to the cells or the membrane prepared in the substantially same  
manner as the one used in the step (a), and

(c) comparing a functional response in the step (a) and one in the step (b) to determine  
10 whether the functional response is reduced by the test compound.

In this screening method of the binding assay, the agonist can be selected from  
zaprinast, an analogue thereof, or a mimetic thereof.

Another aspect of the present invention is use of zaprinast, an analogue thereof, or  
a mimetic thereof as a modulator of a GPR35 protein.

15 Another aspect of the present invention is an isolated and/or purified polypeptide  
of one of the following,

(a) a polypeptide having the amino acid sequence of sequence ID. 2,

(b) a polypeptide having the same amino acid sequence and the same kind of activity as the  
polypeptide of (a) except that one or more amino acids are deleted, substituted, or added,

20 (c) a polypeptide having an amino acid sequence that has at least 86% identity to the  
polypeptide of (a),

(d) a polypeptide having an amino acid sequence that has at least 90% identity to the  
polypeptide of (a), and

(e) a polypeptide having an amino acid sequence that has at least 98% identity to the  
25 polypeptide of (a).

Another aspect of the present invention is a transformed cells that express the  
above mentioned polypeptide and an antibody immunospecific for the above mentioned  
polypeptide.

Another aspect of the present invention is an isolated and/or purified  
30 polynucleotide that encodes the above mentioned polypeptide.

Another aspect of the present invention is a an isolated and/or purified polynucleotide of one of the following,

- (a) a polynucleotide having the nucleotide sequence of sequence ID. 1,
- (b) a polynucleotide that is capable of hybridizing under stringent conditions to a polynucleodide having a nucleotide sequence complementary to the nucleotide sequence of the polynucleotide of (a) and that encodes a polypeptide having the same kind of activity as the polypeptide encoded by the polynucleotide of (a),
- (c) a polynucleotide having a nucleotide sequence that has at least 90% identity to the polynucleotide of (a),
- 10 (d) a polynucleotide having a nucleotide sequence that has at least 95% identity to the polynucleotide of (a), and
- (e) a polynucleotide having a nucleotide sequence that has at least 98% identity to the polynucleotide of (a).

Another aspect of the present invention is an expression vector containing the above mentioned polynucleotide.

Another aspect of the present invention is transformed cells that stably express any of following polypeptides;

- (a) a polypeptide having the amino acid sequence of sequence ID. 2, 4, or 6,
- (b) a polypeptide having the same amino acid sequence and the same kind of activity as the polypeptide of (a) except that one or more amino acids are deleted, substituted, or added,
- 20 (c) a polypeptide having an amino acid sequence that has at least 80% identity to the polypeptide of (a),
- (d) a polypeptide having an amino acid sequence that has at least 90% identity to the polypeptide of (a), and
- 25 (e) a polypeptide having an amino acid sequence that has at least 98% identity to the polypeptide of (a).

Another aspect of the present is a novel compounds that can be identified by any of the above mentioned screening method.

Another aspect of the present is a medicament for use to modulateneural activity comprising a compound that can be identified by any of the above mentioned screening

method. In particular, such a compound can be selected from the group consisting of the following compounds;

2-methyl-5-phenyl-pyrazolo[1,5-a]pyrimidin-7(4H)-one,

3-(4,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl)-4-ethoxy-benzenep  
5 ropanoic acid,

3-[3-(4,5-dihydro-3-methyl-4-oxo-1-propyl-1H-pyrazolo[3,4-d]pyrimidin-6-yl)-4-propoxypheny  
l]-2-propenoic acid,

2,4-dihydro-2-methyl-5-[2-(2-methylpropoxy)-5-(1H-tetrazol-5-yl)-3-pyridinyl]-3-propyl-7H-P  
yrazolo[4,3-d]pyrimidin-7-one,

10 2,4-dihydro-2-methyl-5-[2-(2-methylpropoxy)-5-(1H-1,2,3-triazol-4-yl)-3-pyridinyl]-3-propyl-7  
H-Pyrazolo[4,3-d]pyrimidin-7-one,

5-(2-ethoxyphenyl)-1,4-dihydro-7H-1,2,3-Triazolo[4,5-d]pyrimidin-7-one,

3-(4,7-dihydro-7-oxo-1H-1,2,3-triazolo[4,5-d]pyrimidin-5-yl)-4-propoxy-benzenesulfonyl  
chloride,

15 3-(4,7-dihydro-7-oxo-1H-1,2,3-triazolo[4,5-d]pyrimidin-5-yl)-4-propoxy-benzenesulfonamide,  
5-Nitro-2-(3-phenylpropylamino)benzoic acid,

2-Cyano-4-hydroxyindole,

2-(2-Propoxyphenyl)-8-trifluoromethylpurin-6-one, and

6-phenyl-1-(phenylmethyl)-1H-Bis[1,2,3]triazolo[1,5-a:4',5'-e]pyrimidin-4(5H)-one.

20 Another aspect of the present invention is use of a compound that is identifiable  
by any of the above mentioned screening method.

Another aspect of the present invention is a method of modulating neural activity  
by administering a compound that can be identified by any of the above mentioned screening  
method.

### Definitions

The terms "homologue" as used herein with regard to polypeptide refers to a  
polypeptide having a certain homology with a reference polypeptide. Such a homologue can  
have a homology of preferably, 80% or 85%, more preferably, 90% or 95%, even more  
30 preferably 98%. Such a homologue includes a polypeptide having otherwise the same

amino acid sequence as a reference polypeptide except that one or more (preferably, several) amino acids are added/substituted/deleted. Such a homologue includes a fragment of a polypeptide.

The terms "homologue" as used herein with regard to polynucleotides refers to a polynucleotide having a certain homology with a reference polynucleotide. Such a homologue can have a homology of preferably, 80% or 85%, more preferably, 90% or 95%, even more preferably 98%. Such a homologue includes a polynucleotide that encodes a homologue of a polypeptide that a reference polynucleotide encodes. Such a homologue also includes a polypeptide that is complementary to a polynucleotide capable of hybridizing under stringent conditions (e.g. 65°C and 0.1×SSC {1×SSC=0.15M NaCl, 0.015M Na<sub>3</sub> citrate pH7.0}) to a reference polynucleotide. Such a homologue also includes a polynucleotide fragment or partial polypeptide.

Such sequence homology (identity) can be easily assessed by publicly or commercially available bioinformatics software, such as Blast2 (Altschul, S.F. et al (1997) Nucl. Acids Res. 25, 3389-3402), or programs included in the GCG software package (Devereux et al (1984) Nucl. Acids Res. 12, 387; Wisconsin Package Version 10, Genetics Computer Group (GCG, Madison, Wisconsin), such as Bestfit or Gap.

The term "analogue" as used herein relates to any substance which is similar in structure to a reference agent (e.g. Zaprinst). As used herein, the term "mimetic" relates to any substance which has the same kind of activity or effect as a reference agent (e.g. Zaprinst).

The term "modulator of a receptor" as used herein refers to any substance which has an direct or indirect effect on the receptor. The term "agonist of a receptor" refers to a substance which can stimulate the receptor. The term "antagonist of a receptor" refers to a substance which can inhibit the stimulation of the receptor by an agonist.

The term "functional response" as used herein refers to the reaction that, for example, stimulation of a receptor leads to in cells. In the case of G-protein coupled receptors, this can include, for example, a change in the concentration of cyclic AMP, a transient rise in intracellular calcium concentration, or an opening of an ion channel.

The term "compound" as used herein refers to any chemical entity, including but

not limited to a small organic molecule, a peptide, a protein, a modified protein such as a glycoprotein or a lipoprotein, antibodies or fragments thereof, a nucleic acid such as DNA or RNA or modified nucleic acids, such as oligonucleotides with a modified backbone.

The term "isolated and/or purified" polynucleotide/polypeptide as used herein refers to any polynucleotide/polypeptide that is not in a natural condition. Therefore, "isolated and/or purified" polynucleotide includes the case in which only a promoter or an enhancer of an endogenous polynucleotide is modified keeping the polynucleotide intact.

#### Sequence Listing

Sequence IDs. 1 and 2 respectively show a cDNA nucleotide sequence and an amino acid sequence of rat GPR35.

Sequence IDs. 3 and 4 respectively show a cDNA nucleotide sequence and an amino acid sequence of human GPR35.

Sequence IDs. 5 and 6 respectively show a cDNA nucleotide sequence and an amino acid sequence of rat GPR35.

Sequence IDs. 7-23 show nucleotide sequences of primers used in examples.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention is described in further detail below.

##### Screening method

Generally, the screening method of the present invention is; a method of screening for a compound that modulates a GPR35 protein, comprising the step of:

(a) contacting a GPR35 protein or a partial polypeptide thereof with a test compound.

The screening method can be, but is not limited to, a screening method by binding assay or functional assay. Typical but non-limiting example of a modulating compound is an agonist or an antagonist.

The GPR35 protein or the partial polypeptide thereof used in the screening method of the present invention can exist in cells or a membrane fraction. Thus, a sample

of GPR35 may comprise (1) transformed cells prepared as described below, or (2) cells naturally expressing GPR35, or (3) membrane preparations prepared from any cells expressing GPR35, or GPR35 protein enriched or purified from such cells or membranes. The skilled person will be well aware of methods that can be used to enrich or purify GPR35, which include affinity chromatography, size exclusion chromatography, ion exchange chromatography and other methods suitable for the separation of protein from complex mixtures.

Test compounds that are employed in the screening method of the present invention include, for example, peptides, proteins, non-peptide compounds, synthetic compounds, microbial fermentation products, marine organism extracts, plant extracts, cell extracts, and animal tissue extracts. These test compounds may be either novel or known.

Compounds that may be used for screening include, but are not limited to, peptides such as, for example, soluble peptides, including but not limited to members of random peptide libraries; (see, e.g., Lam et al. (1991) Nature 354, 82-84; Houghten et al. (1991) Nature 354, 84-86), and combinatorial chemistry-derived molecular library made of D- and/or L-configuration amino acids, phosphopeptides (including, but not limited to, members of random or partially degenerate, directed phosphopeptide libraries; see, e.g., Songyang et al. (1993) Cell 72, 767-778), antibodies (including, but not limited to, polyclonal, monoclonal, humanized, anti-idiotypic, chimeric or single chain antibodies, and Fab, F(ab)<sub>2</sub> and Fab expression library fragments, and epitope-binding fragments thereof), and small organic or inorganic molecules.

Peptide libraries may be used as a source of test compounds that can be used to screen in the methods of the invention. Diversity libraries, such as random or combinatorial peptide or nonpeptide libraries can be screened for molecules that specifically bind to a GPR35 protein. Many libraries are known in the art that can be used, e.g., chemically synthesized libraries, recombinant (e.g. phage display libraries), and *in vitro* translation-based libraries.

Examples of chemically synthesized libraries are described in Fodor et al. (1991) Science 251, 767-773; Houghten et al. (1991), Nature 354, 84-86; Lam et al. (1991), Nature 354, 82-84; Medynski (1994) Bio/Technology 12, 709-710; Gallop et al. (1994), J. Medicinal Chemistry 37, 1233-1251; Ohlmeyer et al. (1993), Proc. Natl. Acad. Sci. USA 90, 10922-10926;

Erb et al. (1994), Proc. Natl. Acad. Sci. USA 91, 11422-11426; Houghten et al. (1992) Biotechniques 13, 412; Jayawickreme et al. (1994), Proc. Natl. Acad. Sci. USA 91, 1614-1618; Salmon et al. (1993) Proc. Natl. Acad. Sci. USA 90, 11708-11712; PCT Publication No. WO 93/20242; and Brenner and Lerner (1992) Proc. Natl. Acad. Sci. USA 89, 5381-5383.

5           Examples of phage display libraries are described in Scott & Smith (1990) Science 249:386-390; Devlin et al. (1990) Science 249, 404-406; Christian, et al. (1992), J. Mol. Biol. 227, 711-718; Lenstra (1992) J. Immunol. Meth. 152, 149-157; Kay et al. (1993) Gene 128, 59-65; and PCT Publication No. WO 94/18318 dated August 18, 1994.

10           By way of example of nonpeptide libraries, a benzodiazepine library (see e.g. Bunin et al. (1994), Proc. Natl. Acad. Sci. USA 91, 4708-4712) can be adapted for use. Peptoid libraries (Simon et al. (1992) Proc. Natl. Acad. Sci. USA 89, 9367-9371) can also be used. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh et al. (1994) Proc. Natl. Acad. Sci. USA 91, 11138-11142).

15           Screening the libraries can be accomplished by any of a variety of commonly known methods. See, e.g., the following references, which disclose screening of peptide libraries: Parmley & Smith (1989) Adv. Exp. Med. Biol. 251, 215-218; Scott & Smith (1990) Science 249, 386-390; Fowlkes et al. (1992) BioTechniques 13, 422-427; Oldenburg et al. (1992) Proc. Natl. Acad. Sci. USA 89, 5393-5397; Yu et al. (1994) Cell 76, 933-945; Staudt et al. 20 (1988) Science 241, 577-580; Bock et al. (1992) Nature 355, 564-566; Tuerk et al. (1992) Proc. Natl. Acad. Sci. USA 89, 6988-6992; Ellington et al. (1992) Nature 355, 850-852; U.S. Patent No. 5,096,815, U.S. Patent No. 5,223,409, and U.S. Patent No. 5,198,346, all to Ladner et al.; Rebar & Pabo (1993) Science 263, 671-673; and PCT Publication No. WO 94/18318.

25           Compounds that can be tested and identified using the methods described herein can include, but are not limited to, compounds obtained from any commercial source, including Aldrich (1001 West St. Paul Ave., Milwaukee, WI 53233), Sigma Chemical (P.O. Box 14508, St. Louis, MO 63178), Fluka Chemie AG (Industriestrasse 25, CH-9471 Buchs, Switzerland (Fluka Chemical Corp. 980 South 2nd Street, Ronkonkoma, NY 11779)), Eastman Chemical Company, Fine Chemicals (P.O. Box 431, Kingsport, TN 37662), 30 Boehringer Mannheim GmbH (Sandhofer Strasse 116, D-68298 Mannheim), Takasago (4

Volvo Drive, Rockleigh, NJ 07647), SST Corporation (635 Brighton Road, Clifton, NJ 07012), Ferro (111 West Irene Road, Zachary, LA 70791), Riedel deHaen Aktiengesellschaft (P.O. Box D-30918, Seelze, Germany), PPG Industries Inc., Fine Chemicals (One PPG Place, 34th Floor, Pittsburgh, PA 15272). Further any kind of natural products may be screened using the methods of the invention, including microbial, fungal, plant or animal extracts.

Furthermore, diversity libraries of test compounds, including small molecule test compounds, may be utilized. For example, libraries may be commercially obtained from Specs and BioSpecs B.V. (Rijswijk, The Netherlands), Chembridge Corporation (San Diego, CA), Contract Service Company (Dolgoprudny, Moscow Region, Russia), Comgenex USA Inc. (Princeton, NJ), Maybridge Chemicals Ltd. (Cornwall PL34 OHW, United Kingdom), and Asinex (Moscow, Russia).

Still furthermore, combinatorial library methods known in the art, can be utilized, including, but not limited to: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one-compound" library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam (1997) Anticancer Drug Des. 12, 145). Combinatorial libraries of test compounds, including small molecule test compounds, can be utilized, and may, for example, be generated as disclosed in Eichler & Houghten (1995) Mol. Med. Today 1, 174-180; Dolle (1997) Mol. Divers. 2, 223-236; and Lam (1997) Anticancer Drug Des. 12, 145-167.

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt et al. (1993) Proc. Natl. Acad. Sci. USA 90, 6909; Erb et al. (1994) Proc. Natl. Acad. Sci. USA 91, 11422; Zuckermann et al. (1994) J. Med. Chem. 37, 2678; Cho et al. (1993) Science 261, 1303; Carrell et al. (1994) Angew. Chem. Int. Ed. Engl. 33, 2059; Carrell et al. (1994) Angew. Chem. Int. Ed. Engl. 33, 2061; and Gallop et al. (1994) J. Med. Chem. 37, 1233.

Libraries of compounds may be presented in solution (e.g. Houghten (1992) Bio/Techniques 13, 412-421), or on beads (Lam (1991) Nature 354:82-84), chips (Fodor (1993) Nature 364, 555-556), bacteria (U.S. Patent No. 5,223,409), spores (Patent Nos. US 5,571,698;

US 5,403,484; and US 5,223,409), plasmids (Cull et al. (1992) *Proc. Natl. Acad. Sci. USA* 89, 1865-1869) or phage (Scott and Smith (1990) *Science* 249, 386-390; Devlin (1990) *Science* 249, 404-406; Cwirla et al. (1990) *Proc. Natl. Acad. Sci. USA* 87, 6378-6382; and Felici (1991) *J. Mol. Biol.* 222, 301-310).

- 5           Screening the libraries can be accomplished by any of a variety of commonly known methods. See for example the following references, which disclose screening of peptide libraries: Parmley & Smith (1989) *Adv. Exp. Med. Biol.* 251, 215-218; Scott & Smith (1990) *Science* 249, 386-390; Fowlkes et al. (1992) *BioTechniques* 13, 422-427; Oldenburg et al. (1992) *Proc. Natl. Acad. Sci. USA* 89, 5393-5397; Yu et al. (1994) *Cell* 76, 933-945; Staudt et al. (1988) *Science* 241, 577-580; Bock et al. (1992) *Nature* 355, 564-566; Tuerk et al. (1992) *Proc. Natl. Acad. Sci. USA* 89, 6988-6992; Ellington et al. (1992) *Nature* 355, 850-852; U.S. Patent No. 5,096,815, U.S. Patent No. 5,223,409, and U.S. Patent No. 5,198,346, all to Ladner et al., Rebar & Pabo (1993) *Science* 263, 671-673; and PCT Publication No. WO 94/18318.

Another aspect of the present invention is:

- 15   a method of screening for a compound that inhibits the binding of a ligand to a GPR35 protein, comprising the steps of:
- (a) contacting a GPR35 protein or a partial polypeptide thereof with the ligand,
  - (b) contacting a GPR35 protein or a partial polypeptide thereof prepared in the substantially same manner as the one used in the step (a) with the ligand prepared in the substantially
  - 20   same manner as the one used in the step (a), in the presence of a test compound, and
  - (c) comparing the results of the step (a) and the step (b) to determine whether the binding of the ligand is affected by the presence of the test compound.

In this screening method of the binding assay, a detectable label can be bound to the ligand, and the step (c) can comprise the step of comparing the detected amount of label in the step (a) and the detected amount of label in the step (b). For example, ligands labeled with  $^{125}\text{I}$ ,  $^{35}\text{S}$ ,  $^3\text{H}$ ,  $^{14}\text{C}$ , ligands labeled with fluorescent compounds such as fluorescein, or ligands labeled with enzymes such as horseradish peroxidase can be used as a labeled ligand. Alternatively, these labeled ligands can be prepared according to per-se known methods.

- Another aspect of the screening method of the present invention is a screening
- 30   method for agonists or antagonists of GPR35 by functional assay.

One aspect of the agonist screening of the present invention is;

a method of screening for a compound that is an agonist of a GPR35 protein, comprising the steps of

- (a) adding a test compound to cells expressing a GPR35 protein or a partial polypeptide thereof or to a membrane fraction from the cells, and
- (b) determining whether a functional response is observed.

One aspect of the antagonist screening of the present invention is;

a method of screening for a compound that is an antagonist of GPR35 protein, comprising the steps of

- (a) adding an agonist to cells expressing GPR35 protein or a partial polypeptide thereof or to a membrane fraction from the cells,
- (b) adding an agonist prepared in the substantially same manner as the one used in the step (a) and a test compound to the cells or the membrane prepared in the substantially same manner as the one used in the step (a), and
- (c) comparing a functional response in the step (a) and one in the step (b) to determining whether the functional response is reduced by the test compound.

With regard to the agonist/antagonist screening method described above, in a

preferred aspect of the invention, the functional response can be a transient rise in

intracellular calcium concentration, measured by using fluorescent dyes such as Fluo-3 or

- Indo-1 or by other means known to the person skilled in the art; in another preferred aspect of the invention, the functional response is an increase in the rate of respiration of the cells as measured by an increase in the rate of acidification of the medium surrounding the cells as measured by microphysiometry. In yet another preferred aspect of the invention, the functional response is an increase or decrease in the cyclic AMP concentration or increase in the calcium concentration in the cells, as measured e.g. by increased activity of a reporter gene product wherein the coding region of the reporter gene is functionally linked to a promoter comprising at least one cyclic AMP response element or one element that can respond to the signal transmission that is associated with the increase of the intracellular calcium concentration. Other methods to measure changes in cyclic AMP concentration or calcium concentration in cells are well known to the person skilled in the art. In a further

preferred aspect of the invention, cells are transfected with a plasmid or plasmids leading to co-expression of GPR35 and GFP- $\beta$ -arrestin complex, and an agonist of the receptor is identified by observing clustering of fluorescence, i.e. GFP- $\beta$ -arrestin complex, on the cell surface. Suitable cells expressing GPR35 can be, for example, selected from cells naturally  
5 expressing GPR35, cells where the expression of GPR35 has been upregulated, or cells that has been transfected to express GPR35.

### Transfection

The transfectants of the present invention can be prepared by the following  
10 non-limiting methods.

A cDNA or genomic DNA that encodes the GPR35 protein can be used to construct an expression vector that comprises a DNA encoding the GPR 35 protein. A DNA that encodes a partial polypeptide having the substantially same kind of ligand-binding activity as the GPR35 protein can also be used. A cDNA or genomic DNA that encodes a known or  
15 novel GPR35 protein can be used as well as a synthetic DNA. Specifically, for example, a DNA that encodes the GPR35 protein having the amino acid sequence represented by SEQ ID No. 2, 4, or 6 can be used. An example of such a DNA is a DNA having a base sequence represented by SEQ ID No.1, 3, or 5. These DNAs can also be prepared by known gene engineering techniques.

As an expression vector, for example, pcDNA3.1, pAKKO-111, pAKKO-111H, pXT1, pRC/CMV, pRC/RSV, etc. can be used. As a promoter, any one which functions efficiently in a host cell can be used, for example, SV40 promoter, CMV promoter, HSV-TK promoter, SR $\alpha$  promoter, RSV promoter, etc. can be used. In addition, it is preferable to use the expression vector further containing an enhancer, splicing signal, poly A addition signal,  
25 selective marker, etc. The selective marker includes dihydrofolate reductase genes, neomycin resistance genes (G418 resistance), and the like.

The preferred expression vector holding a DNA encoding the GPR35 protein of the present invention is a vector that is inserted with an above-mentioned promoters (in particular, SR $\alpha$  promoter, CMV promoter, RSV promoter, etc.) upstream of the DNA  
30 encoding the GPR35 protein, a poly A addition signal downstream of the DNA, a selective

marker such as neomycin resistance gene or DHFR gene downstream of the poly A addition signal, and an ampicillin resistance gene downstream of the selective marker. More specifically, an expression vector that comprises a CMV promoter upstream of a DNA that encodes human, rat or mouse GPR35 protein, a poly A addition signal downstream of the DNA, a neomycin resistance gene downstream of the poly A addition signal, and an ampicillin resistance gene downstream of the selective marker is preferable. By the introduction of thus obtained expression vector holding a DNA encoding the GPR35 protein to a host cell, a cell that highly expresses the GPR35 can be obtained.

As to host cells, human embryonic kidney 293 (HEK293) cells (Exp Physiol, 75(3): 309-319 (1990)) and CHO cells (J. Exp. Med., 108: 945 (1995)) are preferred. The HEK293 cells and CHO cells can not only highly express the GPR35 protein but also remarkably stably express it. Preferable combination of an expression vector and host cells can be optimally chosen. For example, the combination of the expression vector represented by hGPR35/pcDNA3.1 and the CHO cells is preferable. For the introduction of an expression vector to host cells, any known method such as calcium phosphate transfection (Virology, 52: 456-467 (1973)), electroporation (EMBO J., 1: 841-845 (1982)), commercially available transfection reagents (for example, FuGENE6 Transfection Reagent by Roche), and the like can be used.

The cells highly expressing the human GPR35 receptor protein may be prepared by first selecting a transformant from the cells transfected with the above-mentioned expression vector using a selective marker as an indicator, followed by clonal selection. When a neomycin resistant gene is used, the resistant cells may be selected by continuous incubation with addition of G418 to multiply the transgene in the cells, thereby yielding much more expressing cells.

In the incubation of the cells (host cells and transfected cells (regardless of transient or stable expression)) used in the present invention, 0.5-20% fetal calf serum EMEM, DMEM, RPMI1640, MEM- $\alpha$ , and the like may be used. Particularly, when an expression vector containing a neomycin gene as a selective marker is used, it is preferable to use a DMEM medium containing G418/geneticin/neomycin and fetal calf serum. The preferred pH is at approximately 6 to 8. The incubation is usually carried out at about

30-40°C for a period of about 15 to 200 hours, if required with aeration, agitation, and exchange of the culture medium.

Despite considerable difficulties, the Inventors finally constructed animal cells that continuously and stably express GPR35 after all their commitment and effort. Such cells are indispensable research tool for high throughput and large scale screening (for example, HTS). Preparation of such a transfectant is explained in detail in EXAMPLES.

#### Antibody

The present invention further provides antibodies immunospecific for the rat GPR35 protein. The antibodies may be obtained by administering the polypeptides or epitope-bearing fragments, analogs or cells to an animal, preferably a non-human animal, according to known techniques. For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler, G. and Milstein, C., Nature (1975) 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozboret al., Immunology Today (1983) 4:72) and the EBV-hybridoma technique (Cole et al., Monoclonal Antibodies and Cancer Therapy 77-96, Alan R. Liss, Inc., 1985). Techniques for the production of single chain antibodies, such as those described in U.S. Pat. No. 4,946,778, can also be adapted to produce single chain antibodies to polypeptides of this invention. Also, transgenic mice, or other organisms, including other mammals, may be used to express humanized antibodies. The above-described antibodies may be employed to isolate or to identify clones expressing the polypeptide or to purify the polypeptides by affinity chromatography. Antibodies against polypeptides of the present invention may also be employed to treat the diseases, amongst others.

#### Medical Indication

Compounds of the present invention (namely, compounds that modulated GPR35 protein (GPR modulator)) can be used to modulate GPR 35 protein and hence modulate neurotransmission in the nervous systems (or spinal cord) so as to treat a variety of neurological disorders (for example, CNS disorders). Non limiting examples of such

conditions are pain, anxiety, convulsions, cognition disorders, obesity, schizophrenia, neurodegeneration, depression, attention deficit hyperactivity disorder, mania, memory deficit, eating disorders, Parkinson's disease, Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis, drug addiction, bipolar disorders, circadian rhythm disorders, migraine, sexual dysfunctions, sleep disorders and eating disorders, urinary diseases, etc.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g. for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds that exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosages for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED 50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (i. e. the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients.

Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or

the nose) or oral, buccal, parenteral or rectal administration.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g. pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g. lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g. magnesium stearate, talc or silica); disintegrants (e.g. potato starch or sodium starch glycolate); or wetting agents (e.g. sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g. sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g. lecithin or acacia); non-aqueous vehicles (e.g. almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g. methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, e. g.

by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g. in ampules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g. sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g. containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

### EXAMPLES

The present invention is explained more specifically by examples below, which do not limit the scope of the invention.

#### Example 1: Original Cloning of human GPR35

A full-length cDNA of human GPR35 was isolated from human Standard Mixed DRG cDNA library (LIFE TECHNOLOGIES; 11649-019, Lot No: 80160b) based upon the known sequence of human GPR35 protein using very well-known technology. Compared with the published sequence of GPR35 (accession number: AF027957), the clone had three missense mutations (T108M, R174A, and R294S) that seemed to be SNPs (the substitutions

were registered in databases independently. See also Nat. Genet., 26:163-175 (2000)). The nucleotide sequence of the clone is shown in sequence ID. 3 and the amino acid sequence of the polypeptide encoded by the clone is shown in sequence ID. 4. This was used as template DNA for following experiments to construct expression vectors for human GPR35.

5

#### Example 2: Construction of an Expression Vector of FLAG-tagged human GPR35

A cDNA of FLAG-tagged human GPR35 was generated by PCR using two primers of sequence ID. 7 and sequence ID. 8. The former primer contains *Eco*RI site and the latter primer *Xba*I site. The PCR was performed using Advantage-HF2 PCR Kit (CLONTECH; K1914-1) with the following program: 94 °C for 4min; 30 cycles of 94 °C for 20 sec, 50 °C for 30 sec, 72 °C for 90sec; 72 °C for 4 min. The PCR product was purified by High Pure™ PCR Product Purification Kit (Roche 1732 676). The purified PCR product was digested with 20 U of *Eco*RI (NEB; R0101S) and 20 U of *Xba*I (NEB; R0145S) in 1xSuRE Cut Buffer H (Roche) at 37 °C for 2.5 hr. A mammalian expression vector, pcDNA3.1(+) (Invitrogen; V790-20) was also digested in the same manner. The samples were electrophoresed with 1% agarose gel, and desired fragments were gel-purified using DNA PREP (IATRON; AGC-001K). Digested pcDNA3.1(+) was treated with "Alkaline Phosphatase, shrimp" (Roche; 1 758 250 ) at 37 °C for 1 hr. The enzyme was then inactivated at 65 °C for 15min. The treated pcDNA3.1(+) and human FLAG-GPR35 fragments were ligated using Rapid DNA Ligation Kit (Roche; 1 635 379). Then, One Shot TOP10 Chemically Competent *E. coli* (C4040-03) was transformed using 4 µl of the reaction mixture. About 200 transformants were obtained, and 16 colonies were picked up to check by PCR using universal primers, T7 and BGH reverse. Four clones were checked by DNA sequencing. The DNA sequencing was performed by TOYOBO Gene Analysis using T7 and BGH reverse primer, and the data were analyzed by GENETYX-WIN version 4. Finally, two of the properly constructed clones were obtained and used for further experiments. The plasmid map of this vector is shown in Figure 1.

#### Example 3: Construction of an Expression Vector of Native human and mouse GPR35

30 A pcDNA3.1 (+) expression vector containing a cDNA of native (namely,

not-tagged) human GPR35 was constructed by the substantially same method as those described above. Primers used for PCR were the primer of sequence ID. 9 (5' primer) and the primer of sequence ID. 8 (3' primer). The former primer contains *EcoRI* site and the latter primer *XbaI* site. The plasmid map of the vector is shown in Figure 2. Similarly, a pcDNA3.1 (+) expression vector containing a cDNA of native mouse GPR35 was constructed. Primers used for PCR were the primer of sequence ID. 10 and the primer of sequence ID. 11. Mouse bone marrow total RNA was used for cloning.

#### Example 4: Cloning of rat GPR35 and Sequencing thereof

To identify and clone rat GPR35 that has not been published, RT-PCR experiments were performed. To generate first-strand cDNA templates for RT-PCR, 5  $\mu$ g of Rat Colon Total RNA (CLONTECH; #64066-1) was reverse-transcribed by SUPERSCRIPT Preamplification Systems (GIBCO BRL/Invitrogen) according to the attached protocol. To amplify rat GPR35 by RT-PCR, a 5' primer of Sequence ID. 12 and a 3' primer of Sequence ID. 13 was used. These primers were designed according to nucleotide sequences of mouse GPR35 (accession number; BC027429). Mixtures for PCR were prepared using LA Taq with GC Buffer II (TaKaRa; RR02AG) according to 'general reaction mixture for PCR' described in the attached protocol. PCR was performed under the following condition: 94 °C for 4min; 35 cycles of 94 °C for 20 sec, 50 °C for 30 sec, 72 °C for 1 min; 72 °C for 4 min. After PCR, 10  $\mu$ l of the samples were separated by electrophoresis. Although non-specific amplifications were also observed, PCR product with expected size was obtained. The fragment with expected size was gel-purified using DNA Prep (IATRON; AGC-001K) and TA-cloned using TOPO TA Cloning Kit (Invitrogen; K4500-01). Plasmids from the several independent clones were purified using QIAprep Spin Miniprep Kit (QIAGEN; #27106), and they were sequenced by TOYOBO Gene Analysis using T7 primer and M13 reverse primer. Determined sequences were analyzed by GENETYX-SV/R version 5.2 (GENETYX) and BLAST Search. The clones (plasmids) contained 921-bp ORF. The nucleotide sequence of the clone is shown in sequence ID. 1 and the amino acid sequence of the polypeptide encoded by the clone is shown in sequence ID. 2. Analysis by BLAST Search revealed that it encodes polypeptide that is most similar to the mouse and human GPR35. Homology of rat GPR35

was 72% to human GPR35 and 85% to mouse GPR35 in amino acid sequence. Homology of rat GPR35 was 78% to human GPR35 and 89% to mouse GPR35 in nucleic acid sequence. Therefore, we concluded that the 921-bp ORF was rat GPR35 gene. To determine error-free rat GPR35 gene, several independent experiments were performed.

5

#### Example 5: Construction of an Expression Vector of rat GPR35

To prepare insert for mammalian expression vector for rat GPR35, PCR was performed using Advantage HF2 PCR Kit (CLONTECH; K1909-1). TA-cloned error-free rat GPR35 was used as a template for the PCR. Two primers used for the PCR were 5' primer of Sequence ID. 14 and 3' primer of Sequence ID. 15. Conditions for the PCR were as follows: 94 °C for 4 min; 30 cycles of 94 °C for 20 sec, 50 °C for 30 sec, 72 °C for 1 min; 72 °C for 4 min. The fragments in the PCR products were separated by electrophoresis and extracted by DNA Prep. The gel-purified PCR fragments were digested by 20 U of *Eco*RI and 20 U of *Xho*I in 1×SuRECut Buffer H (Roche), and ligated into *Eco*RI-*Xho*I sites of pcDNA3.1(+). Several single clones were checked by DNA sequencing (TOYOBO Gene Analysis). For large-scale preparation of the plasmid, EndoFree Plasmid Maxi Kit (QIAGEN; 12362) was used.

10

15

#### Example 6: Transient Transfection

HEK293 cells were maintained in DMEM (Invitrogen; 11965-084) supplemented with 10% FBS and penicillin/streptomycin. Expression vectors of FLAG-tagged human GPR35 (FLAG-hGPR35), native human GPR35, and rat GPR35 were prepared as described above. These expression vectors as well as expression vectors (cloned in pcDNA3 (Invitrogen)) of chimeric G proteins (Gqs5, Gqi5 and Gqo5) and G $\alpha$ 16. were used for transfection of HEK293 cells. Transfection of HEK293 cells was performed using FuGENE 6 Transfection Reagent (Roche; 814 443) according to the attached protocol.

20

25

#### Example 7: Stable Transfection

Commercially available CHO-G $\alpha$ 15-NFAT  $\beta$ -lactamase cells were maintained in DMEM (Invitrogen 11995-065) supplemented with 10% FBS (Biowhittaker 14-502F), 0.1

30

mM NEAA (Invitrogen 11140-050), 1.0 mM Na-pyruvate (Invitrogen 11360-070), 25 mM HEPES (Invitrogen 15630-080), 3 µg/ml blasticidin HCl (Invitrogen R210-01), 100 µg/ml zeocin (Invitrogen R250-01) and penicillin/streptomycin (Invitrogen 15140-122). About  $6 \times 10^5$  cells were transfected with 4.5 µg of human GPR35-expression vector using 15 µl of Lipofectamine 2000 (Invitrogen 11668-019). One-day after transfection, the cells were harvested and re-suspended in a medium prepared by further supplementing DMEM above with 1mg/ml G418 (Invitrogen 11811-031). The cells were seeded into T75 flask (Corning 430641) at a density of  $1 \times 10^6$  cells/flask and incubated for 11 days in CO<sub>2</sub> incubator. The stable cells were stimulated with 10 µM of zaprinast (Sigma Z0878) overnight, and selection of zaprinast-sensitive cells was performed on Fluorescence Activated Cell Sorter (FACSVantage SE; Becton Dickinson). For sorting, a substrate of β-lactamase was prepared by mixing CCF4 (Invitrogen K1028) with pluronic F127 (Invitrogen K1026), PBS (-) (Invitrogen 14190-144) and probenecid (Sigma P8761) just before use. The response to zaprinast on each sorted cell was confirmed by β-lactamase assay and Ca assay. Thus, stably-expressing human GPR35 cell line was established.

#### Example 8: Intracellular Calcium Mobilization Assay

Two days after transfection, the cells were washed twice with Hanks' Balanced Salt Solution (GIBCO BRL; 14175-095), and loaded with 5 µM Fura 2-AM (DOJINDO; 343-05401) in Hanks' Balanced Salt Solution containing Pluronic F-127 (0.05 %v/v, SIGMA; P-2443) for 1 hour in CO<sub>2</sub> incubator. After the incubation, the cells were harvested by centrifugation and suspended in Hanks' Balanced Salt Solution (GIBCO BRL; 14025-092) (this solution contains calcium chloride, magnesium chloride, and magnesium sulfate) at a density of  $1\text{--}3 \times 10^5$  cells/ml. Cell suspension (90 µl) was dispensed to 96-well plates (COSTER; 3904). Addition of 10 µl of the test compounds and measurement of intracellular calcium mobilization were performed with FDSS6000 (Hamamatsu Photonics).

#### Example 9: Western Blotting

To confirm expression of FLAG-hGPR35,  $1.5 \times 10^6$  cells were dissolved in 150 µl of Tris-SDS-BME Sample Loading Buffer (Owl; ER33) and analyzed by western blotting

analysis. 20  $\mu$ l of the samples were loaded to READY GELS J (BIO-RAD; 161J391) and separated by electrophoresis in Tris/Glycine/SDS Buffer (BIO-RAD; 161-0732) at 200 volts for 30 min. Proteins were blotted to PVDF membrane (MLIIPORE; IPVH304FO) at 15 V for 30min, and the blotted membrane was blocked by TBS buffer (150 mM NaCl, 20 mM Tris-HCl; pH7.5) containing 1.2 % bovine albumin (SIGMA; A-7030) for 30min. The membranes were incubated in 1,000-fold diluted ANTI-FLAG M2 Monoclonal Antibody (SIGMA; F-3165) for 1 hour and washed with TTBS buffer (TBS buffer containing 0.1% Tween20) three times. Membranes were incubated in 500-fold diluted anti-mouse IgG-HRP (IMMUNOTECH; 0817) as secondary antibody and washed with TTBS buffer three times. FLAG-GPR35 was visualized by ECL Western blotting detection reagents (Amersham pharmacia biotech; RPN 2209), and the signals were detected by IMAGE READER LAS-1000 Pro version 2.0 (FUJI PHOTO FILM).

#### Example 10: Screening for Agonists for human GPR35

To screen agonists for GPR35, we prepared HEK293 cells transiently co-expressing FLAG-tagged human GPR35 (FLAG-hGPR35), chimeric G proteins (Gqs5, Gqi5 and Gqo5) and a promiscuous G protein (G $\alpha$ 16). After the cells were loaded with Fura 2-AM (calcium indicator), intracellular Ca<sup>2+</sup> mobilization evoked by addition of 10  $\mu$ M of compounds was detected with FDSS6000 by the method described above. About 1,000 compounds were screened, and we discovered that zaprinast induced intracellular Ca<sup>2+</sup> mobilization in the co-transfectant. It had no such effect observed in parental HEK293 cells.

As a result of the screening of various compounds, besides Zaprinast, the following compounds were identified to induce intracellular Ca<sup>2+</sup> mobilization in the co-transfectant.

Identified compound (reference)	EC50( $\mu$ M)
2-methyl-5-phenyl-pyrazolo[1,5-a]pyrimidin-7(4H)-one (Journal of Organic Chemistry (1959), 24, 779-86)	2
3-(4,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl)-4-ethoxy-benz enepropanoic acid (Cell Pathways, Inc., USPAT No. 6200980)	~3

3-[3-(4,5-dihydro-3-methyl-4-oxo-1-propyl-1H-pyrazolo[3,4-d]pyrimidin-6-yl)-4-propoxyphenyl]-2-propenoic acid (Cell Pathways, Inc., USPAT No. 6200980)	0.80
2,4-dihydro-2-methyl-5-[2-(2-methylpropoxy)-5-(1H-tetrazol-5-yl)-3-pyridinyl]-3-propyl-7H-Pyrazolo[4,3-d]pyrimidin-7-one (Pfizer Limited, WO 2001027112)	1.6
2,4-dihydro-2-methyl-5-[2-(2-methylpropoxy)-5-(1H-1,2,3-triazol-4-yl)-3-pyridinyl]-3-propyl-7H-Pyrazolo[4,3-d]pyrimidin-7-one (Pfizer Limited, WO 2001027112)	0.50
5-(2-ethoxyphenyl)-1,4-dihydro-7H-1,2,3-Triazolo[4,5-d]pyrimidin-7-one (May and Baker Ltd., USPAT No. 3987160)	1.4
3-(4,7-dihydro-7-oxo-1H-1,2,3-triazolo[4,5-d]pyrimidin-5-yl)-4-propoxy-benzenesulfonyl chloride (May and Baker Ltd., USPAT No. 3987160)	0.30
3-(4,7-dihydro-7-oxo-1H-1,2,3-triazolo[4,5-d]pyrimidin-5-yl)-4-propoxy-benzenesulfonamide (May and Baker Ltd., USPAT No. 3933822)	0.68
5-Nitro-2-(3-phenylpropylamino)benzoic acid (Hoechst A.-G., USPAT No. 4994493)	2
2-Cyano-4-hydroxyindole (Leland Stanford Junior University, USPAT No. 5229412)	2
2-(2-Propoxyphenyl)-8-trifluoromethylpurin-6-one (Smith Kline and French Laboratories Ltd., USPAT No. 5073559)	0.30
6-phenyl-1-(phenylmethyl)-1H-Bis[1,2,3]triazolo[1,5-a:4',5'-e]pyrimidin-4(5H)-one (MDPI Product List, commercially available MDPI)	0.24

Table 1: identified compounds

To confirm the agonistic activity of zaprinast, we tested dose dependencies of the responses in the co-transfectants by the same method. In the co-transfectant, intracellular  $\text{Ca}^{2+}$  mobilization was induced by the compound dose-dependently, while HEK293 cells co-expressing only the chimeric G proteins and  $\text{G}\alpha_{16}$  (mock) showed no response (Figure 3) to the compounds. The expression of FLAG-hGPR35 in the co-transfectant was confirmed by western blotting (data not shown). We also confirmed the agonism of zaprinast for native hGPR35 (Figure 3).

#### Example 11: Confirmation of the Coupling of GPR35

We also examined which G protein(s) was/were coupled to GPR35 using zaprinast.

When FLAG-hGPR35 was co-expressed with Gqi5, Gqo5 (these are commercially available) or G $\alpha$ 16 in HEK293 cells, intracellular calcium mobilization was induced by zaprinast (Figure 4). On the other hand, the response was not observed in either co-transfectant of Gqs5 and FLAG-hGPR35 or transfectant of FLAG-hGPR35 alone (Figure 2). The expression of FLAG-hGPR35 in the co-transfectant was confirmed by western blotting (data not shown). These data suggest that human GPR35 couples to G $\alpha$ i, G $\alpha$ o, and G $\alpha$ 16.

#### Example 12: Intracellular Calcium Mobilization Assay using rat GPR35

To confirm agonist activity of zaprinast for rat GPR35, HEK293 cells transiently co-expressing rat GPR35 and Gqi5 (chimeric G protein) were prepared, and challenged with zaprinast by a method described above (Figure 5). Zaprinast showed a moderate agonistic activity for human GPR35 (mean EC<sub>50</sub> value = 1136 nM) but showed surprisingly high agonistic activity for rat GPR35 (mean EC<sub>50</sub> value = 21 nM). Transfectant expressing Gqi5 alone (mock) did not respond to zaprinast (Figure 5). Transfectant expressing GPR35 alone also did not respond to zaprinast (data not shown).

#### Example 13: Comparative Experiment to Confirm Mechanism

Zaprinast is a well-known PDE5 inhibitor (BIOORG MED CHEM LETT (1996) 6 (15), 1819-1824 and EUR J PHARMACOL (2001) 411, 1-10). In the experiments above, Zaprinast showed its activity only when a GPR35 protein and an appropriate G protein are co-expressed. Therefore, it is understood that the above activity of Zaprinast is mediated by GPR35. However, just for further clarification, self-developed compound (hereinafter referred to as compound A) that is a strong PDE5 inhibitor (its chemical formula is 3-ethyl-5-{5-[(4-ethylpiperazino)sulphonyl]-2-propoxyphenyl}-2-(2-pyridylmethyl)-6,7-dihydro-2H-pyrazolo[4,3-d]pyrimidin-7-one. IC<sub>50</sub> value against PDE5 is 540nM for zaprinast and only 0.8nM for the compound A. Compound A is disclosed in Xenobiotica (2001), 31(8-9), 651-664 and 665-676) was used for the calcium mobilization assay. As a result, the compound A, unlike Zaprinast did not show an agonistic activity (Figure 6). This confirms that Zaprinast showed the activity via GPR35 protein. This was also confirmed by a comparative experiment that compared an activity of Zaprinast and the compound A using

spinal cord as described below.

#### Example 14: Electrophysiological Experiment

##### Spinal cord preparation

Transversely sliced spinal cord preparation was obtained from an adult rat by a method already described in an article (Nakatsuka, et al. Alteration in synaptic inputs through C-afferent fibers to substantia gelatinosa neurons of rat spinal dorsal horn during postnatal development Neuroscience, 99, 549-556, 2000). Briefly, male adult Sprague-Dawley rats (200-350 g; 7-8 weeks old, SLC) were anesthetized with urethane (1.5 g/kg, i.p.). After laminectomy, lumbosacral spinal cord was removed and transferred into ice cold Krebs solution (1-3°C) pre-equilibrated with 95% O<sub>2</sub> / 5% CO<sub>2</sub>. The composition of the Krebs solution was (in mM): 117 NaCl, 3.6 KCl, 2.5 CaCl<sub>2</sub>, 1.2 MgCl<sub>2</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub> and 11 glucose. The pia was removed after cutting all the ventral and dorsal roots near the root entry zone. The spinal cord was mounted on a microslicer (DTK-3000W, Dosaka EM Co. Ltd., Kyoto, Japan) and then a transverse slice (500 µm thick) was cut. The slice was placed on a nylon mesh in the recording chamber of about 0.5 ml volume. The cut slice was continuously superfused at a flow rate of 18-20ml/min with the Krebs solution in 95% O<sub>2</sub> / 5% CO<sub>2</sub>, pH 7.2, for pre-incubation (longer than 1hr, 36±1 °C). The institutional animal care and use committee approved the experimental procedure.

##### Patch clamp recordings

Substantia gelatinosa (SG) neurons were identified by their location in the preparation by a method in previously-reported article (Yoshimura and, Nishi Blind patch-clamp recordings from substantia gelatinosa in adult rat spinal cord slices: pharmacological properties of synaptic currents, Neuroscience, 53, 519-526, 1993). Briefly, under a binocular stereomicroscope and with transmitted illumination, the SG neurons were clearly discernable as a translucent band. Blind whole-cell recordings were performed from SG neurons with patch pipettes which had tip resistance of 8-10 MΩ when the pipettes were filled with the solution containing (in mM): 135 K-gluconate, 5 KCl, 2 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub>, 5 EGTA, 5 HEPES, and 5 ATP. Each recording was performed on a cell in a fresh slice without prior application of any drugs, except some experiments. Signals were gained with

a patch-clamp amplifier (Axopatch 200B, Axon Instruments). Data were low-pass filtered at 2 kHz, digitized at 333 kHz with an A/D converter, stored and analyzed with a personal computer using the software of pCLAMP (Version 9.0, Axon Instruments), Axograph (Version 4.6.2, Axon Instruments) and Origin (Version 6.1, Microcal). All experiments were performed to isolate excitatory postsynaptic currents (EPSCs) at the holding potential ( $V_H$ ) of -70 mV, at which GABA and glycine-mediated synaptic currents were negligible. Miniature EPSCs (mEPSCs) were recorded in the presence of 2  $\mu$ M tetrodotoxin (TTX).

#### Application of drugs

All tested compounds were applied through the bath solution by exchanging the perfusion solution at a flow rate of 14-18 ml/min. Zaprinast was purchased from Sigma (St. Louis, MO, USA) and TTX was purchased from WAKO (Japan). The above mentioned self developed compound A was provided by medicinal chemists at Pfizer Sandwich Laboratories.

#### Data analysis and statistics

Data analyses were performed with the help of detection program of synaptic currents in the Axograph software. The events that possess the shape of a rapid rise followed by a slow decay and the peak exceeded -3 or -4 pA were identified as mEPSCs to be analyzed. Each selected synaptic current was visually inspected to eliminate detection errors. Changes of the inter-event interval and amplitude of mEPSCs were evidenced with the help of cumulative histograms of these parameters, and ascertained with the Kolmogorov-Smirnov test. For this test,  $P < 0.01$  was taken to be statistically significant. Data were presented as mean  $\pm$  SEM

#### Result: Effect of zaprinast on mEPSCs

Regarding to the effects of zaprinast on mEPSCs in SG neurons of the spinal cord, zaprinast (10  $\mu$ M) inhibited the frequency of the mEPSCs (Fig. 7). Figs. 7B and 7C demonstrate the effects of zaprinast (10  $\mu$ M) on cumulative distribution of the inter-event interval and the amplitude of the mEPSC. Zaprinast significantly inhibited the frequency ( $P < 0.001$ ) of the mEPSCs (7B), while the amplitude of mEPSCs ( $p > 0.01$ ) was not (7C). The kinetics of mEPSCs was not affected by zaprinast (7D). Of 5 cells examined, 4 cells showed significant suppressions of mEPSC frequency in each cells ( $P < 0.01$ , KS-test). Membrane current was not induced by zaprinast up to 100  $\mu$ M ( $n = 2$ , Data not shown).

These data suggest that zaprinast inhibited the excitatory transmission presynaptically.

Result: Mechanism of the inhibition of mEPSC frequency by zaprinast

In order to study whether this inhibition of the mEPSC induced by zaprinast was GPR35 agonism or PDE-5 inhibition, we examined the effect of the compound A above, a selective PDE-5 inhibitor, on the mEPSCs. The frequency and amplitude of mEPSCs were not affected by the compound A of its concentration from 20 nM up to 10 $\mu$ M (Fig. 8). Since this no inhibitory effect of the compound A was also confirmed in the SG neurons whose frequency of mEPSCs was reduced by the application of zaprinast of 10 $\mu$ M (n=2, Data not shown), the compound A and zaprinast are quite different from their mode of action on mEPSCs. These results indicate that zaprinast would exert its inhibitory effect on mEPSCs via GPR35 agonism but not PDE5 inhibition.

Result

As described above, it has been demonstrated that electrophysiological evidence that activation of GPR35 produced suppression in the release of excitatory neurotransmitters from the presynaptic terminals of excitatory interneuron. These presynaptic GPR35 may play an important role in the inhibitory controls of nociceptive transmission at the spinal cord level.

Example 15: Tissue Distribution of human GPR35

Total RNA samples used to synthesize templates are listed in Table 2. Reverse transcription was performed using SUPERScript Preamplification Systems (CLONTECH) according to the attached protocol. To amplify transcripts of human GPR35, two nucleotide primers, the primer of Sequence ID. 16 and the primer of Sequence ID 17 were designed. Premix Taq Ex Taq Version (TaKaRa; RR003A) was used for PCR. PCR was performed in a volume of 20  $\mu$ l under the following program: 94 °C for 4min; 32 cycles of 94 °C for 20 sec, 65 °C for 30 sec, 72 °C for 60 sec; 72 °C for 4 min. The samples were analyzed by electrophoresis using 1% agarose gel. For a positive control, 1 pg of expression vector of FLAG-tagged GPR35 was used. For an internal control, GAPDH was amplified with the same procedure except for annealing temperature (50 °C), number of cycles (25 cycles) and primers (Sequence IDs 18 and 19). To exclude the possibility of contamination of genomic

DNA to the templates, experiments without reverse transcription (in absence of SuperScript II reverse transcriptase) were performed, and no amplifications were observed except for positive control.

Tissue	Maker	Catalogue #	Conc.(µg/µl)
Trachea	CLONTECH	#K4000-1 (Human Panel I)	1.00
Heart	CLONTECH	#K4001-1 (Human Panel II)	1.00
Kidney	GIBCO BRL	11410-016	2.50
Adrenal gland	CLONTECH	64016-1	1.00
Stomach	CLONTECH	#K4000-1 (Human Panel I)	1.00
Small intestine	CLONTECH	#K4001-1 (Human Panel II)	1.00
Colon	CLONTECH	#K4001-1 (Human Panel II)	1.00
Brain	CLONTECH	#K4003-1 (Human Panel IV)	1.00
Cerebellum	CLONTECH	#K4003-1 (Human Panel IV)	1.00
Spinal cord	CLONTECH	#K4003-1 (Human Panel IV)	1.00
Dorsal horn	ABS	custom-made RNA	0.95
DRG	ABS	custom-made RNA	0.76

5 Table 2. List of total RNA used for RT-PCR.

Tissue distribution of GPR35 in human was determined by RT-PCR (Figure 9). Striking signals of GPR35 were observed in spinal cord (whole), (spinal) dorsal horn, DRG, brain, cerebellum, small intestine and colon. On the other hand, little or no signal was  
 10 observed in trachea, heart, kidney, adrenal gland, and stomach. The internal control (GAPDH) was amplified equally with each template. Furthermore, it was confirmed that no amplified products were obtained by experiment without the reverse-transcription reaction (data not shown). These data show that GPR35 is mainly expressed in the nervous system, small intestine and colon.

15

#### Example 16: Tissue Distribution of rat GPR35

Rat DRG and spinal cord were extracted from a 10-week-old pregnant female

Sprague-Dawley rat sacrificed by CO<sub>2</sub> (all animal care and sample collection were approved by the Research Ethics Committee). The tissues were extracted in 1 ml of ISOGEN (Nippon Gene), and total RNAs were purified according to attached protocol. Other total RNAs were purchased from BD Clontech or UNITECH. They are summarized in Table 3.

5

Tissue	Maker	Catalogue #
Brain	CLONTECH (BD)	64075-1
Cerebellum	UNITECH	203
Cerebrum	UNITECH	202
Skeletal muscle	UNITECH	215
Uterus	UNITECH	213
Heart	UNITECH	205
Lung	UNITECH	206
Liver	UNITECH	208
Bladder	CLONTECH (BD)	64077-1
Stomach	UNITECH	207
Small intestine	UNITECH	211
Colon	CLONTECH (BD)	64066-1
DRG	Prepared in this study	
Spinal cord	Prepared in this study	

Table 3. List of total RNAs of rat used in this study

To generate first-strand cDNA templates for RT-PCR, 5 µg of total RNA (listed in Table 1) was reverse-transcribed by SUPERScript Preamplification Systems (GIBCO BRL/Invitrogen) according to the attached protocol. To exclude the possibility of contamination of genomic DNA to the templates, a series of samples without SuperScript II RT (-RT) were prepared in parallel as negative controls. Twentieth of the each reverse-transcribed sample was used as template for subsequent PCR. To amplify rat GPR35 by RT-PCR, two primers (Sequence Ids 20 and 21) were designed according to nucleotide sequences of rat GPR35. Mixtures for PCR were prepared using LA Taq with GC

Buffer (TaKaRa; RR02AG) according to 'general reaction mixture for PCR' described in the attached protocol. PCR for rat GPR35 was performed under the following condition: 94 °C for 4 min; 35 cycles of 94 °C for 20 sec, 58 °C for 30 sec, 72 °C for 1 min; 72 °C for 4 min. For internal controls of reverse transcription, GAPDH was amplified with the same procedure except for cycle number (30 cycles) and primers (Sequence IDs 22 and 23). After PCR, 10 µl of each sample was separated by electrophoresis, and DNAs were visualized by ethidium bromide staining.

To determine tissue distribution of rat GPR35, RT-PCR analysis was performed. Predominant expression in digestive system (colon, small intestine, stomach) and DRG were observed (Figure 10). This result is consistent with a previous study in human GPR35.

#### Example 17: Ligand Binding Assay

Transiently or stably transfected cells expressing GPR35 are harvested by scraping (24-72 hours after the transfection in the case of transient transfection) and resuspended in 50 ml of ice-cold HEPES buffer (pH7.4) (containing 0.32 M Sucrose, 10mM EDTA, 10mM MgCl<sub>2</sub>). The resulting suspension is centrifuged at 48,000 g, 4 °C for 30 minutes. The resulting pellet is resuspended in the same buffer and centrifugation described above is repeated. The resulting pellet (membrane fraction) is suspended in an appropriate volume of an assay buffer and preserved at -80 °C. The protein concentration is determined via Bradford's assay (Biorad), according to the manufacturer's recommendations.

The membrane fraction containing 50 µg protein is then incubated with various potential ligands, radiolabeled to high specific activity, for about 30-120 minutes at room temperature (the optimal conditions, ion concentrations, incubation time and temperature can be determined for each ligand with our undue difficulty according to technical knowledge well known to those skilled in the art). Reaction solution is filtrated by suction filtration using SKATRON cells harvester when the reaction ends. Right after filtration, the filter is washed by the assay buffer three times. Wallac GF/B filter is immersed in 0.3% (v/v) PEI (Polyethyleneimine; Sigma) for 1 hour before use. The GF/B filter is dried after washing and bound radioactivity on Filtermats is determined using a liquid scintillation counter. The specific binding is defined as the difference between total radioactivity bound minus the

radioactivity measured in the presence of an excess of unlabelled ligand. Mock-transfected cells are also measured to assess whether the host cells express receptors for the ligands used endogenously.

## CLAIMS

1. A method of screening for a compound that modulates a GPR35 protein, comprising the step of  
(a) contacting a GPR35 protein or a partial polypeptide thereof with a test compound.
- 5 2. A method of screening for a compound that modulates neural activity, comprising the steps of  
(a) contacting a GPR35 protein or a partial polypeptide thereof with a test compound.
3. A method of screening for a compound that inhibits the binding of a ligand to a GPR35 protein, comprising the steps of  
10 (a) contacting a GPR35 protein or a partial polypeptide thereof with the ligand,  
(b) contacting a GPR35 protein or a partial polypeptide thereof prepared in the substantially same manner as the one used in the step (a) with the ligand prepared in the substantially same manner as the one used in the step (a), in the presence of a test compound, and  
(c) comparing the results of the step (a) and the step (b) to determine whether the binding of  
15 the ligand is affected by the presence of the test compound.
4. The method according to claim 3, wherein  
a detectable label is bound to said ligand, and  
said step (c) comprises the step of comparing the detected amount of label in the step (a) and the detected amount of label in the step (b).
- 20 5. A method of screening for a compound that is an agonist of a GPR35 protein, comprising the steps of  
(a) adding a test compound to cells expressing a GPR35 protein or a partial polypeptide thereof or to a membrane fraction from the cells, and  
(b) determining whether a functional response is observed.
- 25 6. A method of screening for a compound that is an antagonist of GPR35 protein, comprising the steps of  
(a) adding an agonist to cells expressing GPR35 protein or a partial polypeptide thereof or to a membrane fraction from the cells,  
(b) adding an agonist prepared in the substantially same manner as the one used in the step  
30 (a) and a test compound to the cells or the membrane prepared in the substantially same

manner as the one used in the step (a), and

(c) comparing a functional response in the step (a) and one in the step (b) to determine whether the functional response is reduced by the test compound.

7. A method according to claim 5 or 6, wherein said functional response is a transient  
5 rise in intracellular calcium concentration.

8. The method according to claim 3 or 6, wherein said ligand or said agonist is selected from zaprinast, an analogue thereof, or a mimetic thereof.

9. The method according to any one of claims 1-3, wherein the GPR35 protein or the partial polypeptide thereof in the step (a) exists in cells or a membrane fraction.

10. The method according to any one of claims 1-3, 5, and 6 to screen a compound for use  
10 in the treatment or prevention of any of the following conditions:

pain, anxiety, convulsions, cognition disorders, obesity, schizophrenia, neurodegeneration, depression, attention deficit hyperactivity disorder, mania, memory deficit, eating disorders, Parkinson's disease, Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis,  
15 drug addiction, bipolar disorders, circadian rhythm disorders, migraine, sexual dysfunctions, sleep disorders and eating disorders.

11. The method according to any one of claims 1-3, 5, and 6, wherein said test compound consists of a plurality of compounds.

12. A method according to any one of claims 1-3, 5, and 6, wherein said GPR35 protein is  
20 selected from the following:

(a) a polypeptide having the amino acid sequence of sequence ID. 2, 4, or 6,

(b) a polypeptide having the same amino acid sequence as the polypeptide of (a) except that one or more amino acids are deleted, substituted, or added,

(c) a polypeptide having an amino acid sequence that has at least 80% identity to the the  
25 polypeptide of (a),

(d) a polypeptide having an amino acid sequence that has at least 90% identity to the the polypeptide of (a),

(e) a polypeptide having an amino acid sequence that has at least 98% identity to the the polypeptide of (a), and

30 (f) a human GPR35 protein, a mouse GPR35 protein, or a rat GPR35 protein.

13. Use of zaprinast, an analogue thereof, or a mimetic thereof as a modulator of a GPR35 protein.

14. An isolated and/or purified polypeptide of one of the following;

(a) a polypeptide having the amino acid sequence of sequence ID. 2,

5 (b) a polypeptide having the same amino acid sequence and the same kind of activity as the polypeptide of (a) except that one or more amino acids are deleted, substituted, or added,

(c) a polypeptide having an amino acid sequence that has at least 86% identity to the polypeptide of (a),

10 (d) a polypeptide having an amino acid sequence that has at least 90% identity to the polypeptide of (a), and

(e) a polypeptide having an amino acid sequence that has at least 98% identity to the polypeptide of (a).

15. An isolated and/or purified polynucleotide of one of the following;

(a) a polynucleotide having the nucleotide sequence of sequence ID. 1,

15 (b) a polynucleotide that is capable of hybridizing under stringent conditions to a polynucleotide having a nucleotide sequence complementary to the nucleotide sequence of the polynucleotide of (a) and that encodes a polypeptide having the same kind of activity as the polypeptide encoded by the polynucleotide of (a),

20 (c) a polynucleotide having a nucleotide sequence that has at least 90% identity to the polynucleotide of (a),

(d) a polynucleotide having a nucleotide sequence that has at least 95% identity to the polynucleotide of (a),

(e) a polynucleotide having a nucleotide sequence that has at least 98% identity to the polynucleotide of (a), and

25 (f) a polynucleotide that encodes the polypeptide according to the claim 14.

16. An expression vector containing the polynucleotide according to claim 15.

17. Transformed cells that express the polypeptide according to claim 14.

18. An antibody immunospecific for the polypeptide according to claim 14.

19. Transformed cells that stably express any of following polypeptides;

30 (a) a polypeptide having the amino acid sequence of sequence ID. 2, 4, or 6,

(b) a polypeptide having the same amino acid sequence and the same kind of activity as the polypeptide of (a) except that one or more amino acids are deleted, substituted, or added,

(c) a polypeptide having an amino acid sequence that has at least 80% identity to the polypeptide of (a),

5 (d) a polypeptide having an amino acid sequence that has at least 90% identity to the polypeptide of (a), and

(e) a polypeptide having an amino acid sequence that has at least 98% identity to the polypeptide of (a).

20. A novel compound that is identifiable by the method according to any one of  
10 claims 1-3, 5, and 6.

21. A medicament for use to modulate neural activity comprising a compound that is identifiable by the method according to any of claims 1-3, 5, and 6.

22. The medicament according to claim 21 comprising a compound selected from the group consisting of the following compounds:

15 2-methyl-5-phenyl-pyrazolo[1,5-a]pyrimidin-7(4H)-one,

3-(4,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl)-4-ethoxy-benzenep  
ropanoic acid,

3-[3-(4,5-dihydro-3-methyl-4-oxo-1-propyl-1H-pyrazolo[3,4-d]pyrimidin-6-yl)-4-propoxypheny  
l]-2-propenoic acid,

20 2,4-dihydro-2-methyl-5-[2-(2-methylpropoxy)-5-(1H-tetrazol-5-yl)-3-pyridinyl]-3-propyl-7H-P  
yrazolo[4,3-d]pyrimidin-7-one,

2,4-dihydro-2-methyl-5-[2-(2-methylpropoxy)-5-(1H-1,2,3-triazol-4-yl)-3-pyridinyl]-3-propyl-7  
H-Pyrazolo[4,3-d]pyrimidin-7-one,

5-(2-ethoxyphenyl)-1,4-dihydro-7H-1,2,3-Triazolo[4,5-d]pyrimidin-7-one,

25 3-(4,7-dihydro-7-oxo-1H-1,2,3-triazolo[4,5-d]pyrimidin-5-yl)-4-propoxy-benzenesulfonyl  
chloride,

3-(4,7-dihydro-7-oxo-1H-1,2,3-triazolo[4,5-d]pyrimidin-5-yl)-4-propoxy-benzenesulfonamide,

5-Nitro-2-(3-phenylpropylamino)benzoic acid,

2-Cyano-4-hydroxyindole,

30 2-(2-Propoxyphenyl)-8-trifluoromethylpurin-6-one, and

6-phenyl-1-(phenylmethyl)-1H-Bis[1,2,3]triazolo[1,5-a:4',5'-e]pyrimidin-4(5H)-one.

23. Use of a compound that is identifiable by the method according to any one of claims 1-3, 5, and 6 to modulate neural activity.

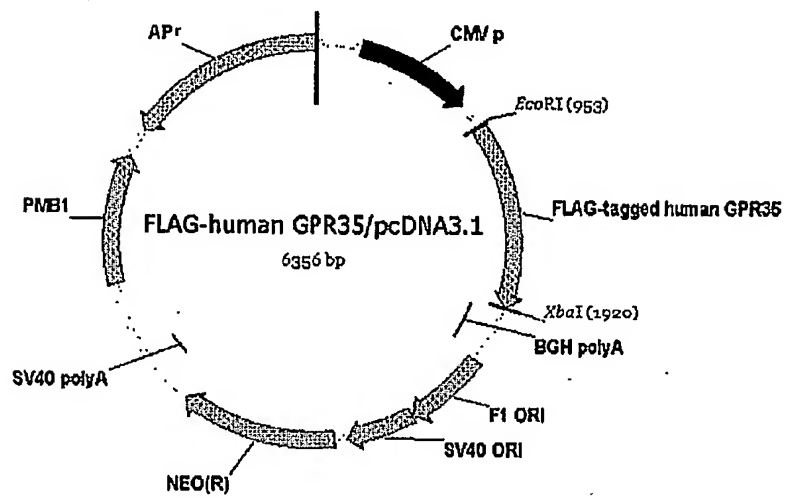
24. Use of a compound that is identifiable by the method according to any one of claims  
5 1-3, 5, and 6 for the manufacture of a medicament to modulate neural activity.

25. A method of modulating neural activity by administering a compound that is identifiable by the method according to any one of claims 1-3, 5, and 6.

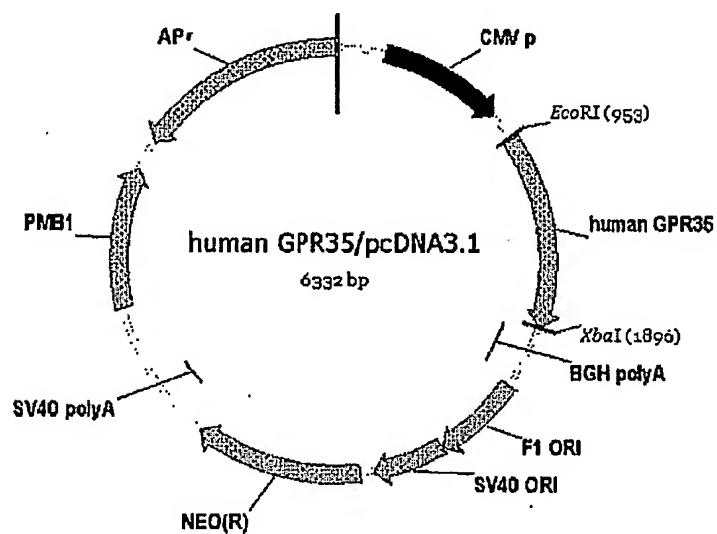
**ABSTRACT**

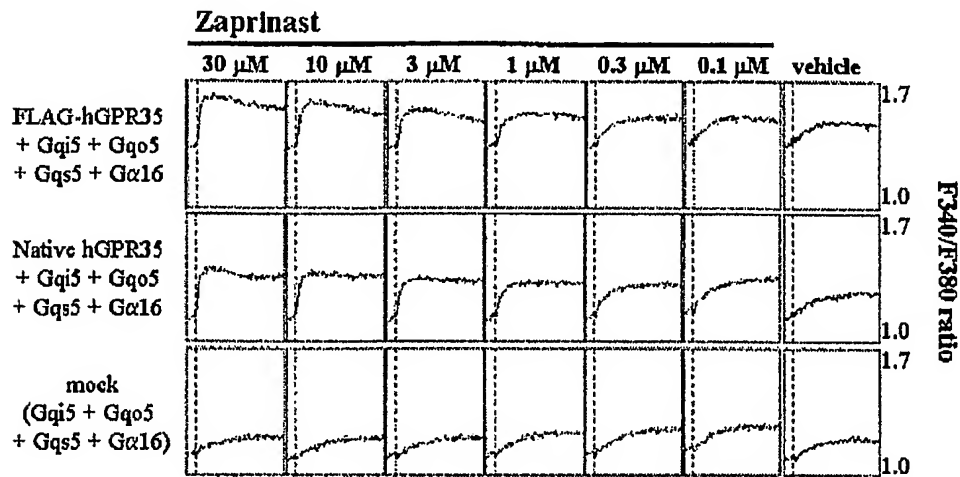
The present invention primarily relates to function analysis of GPR35, an orphan GPCR (G protein coupled receptor), a screening method to identify modulators (agonists/antagonists) of the receptor, cells stably expressing the receptor, and the discovery  
5 of modulators of the receptor. The present invention also relates to novel rat GPR35.

**Figure 1. Expression vector of FLAG-hGPR35**

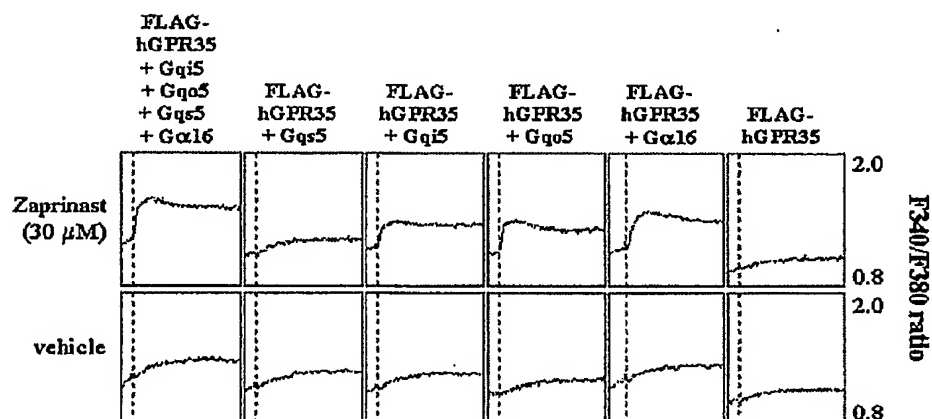


**Figure 2. Expression vector of native-hGPR35**

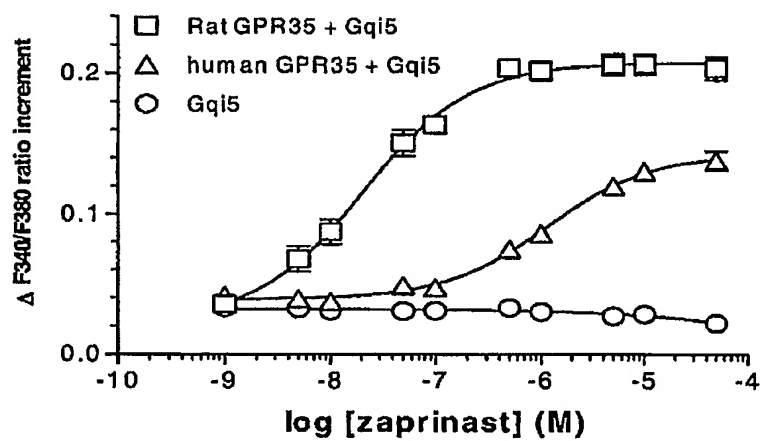


**Figure 3. Dose dependent effects of zaprinast**

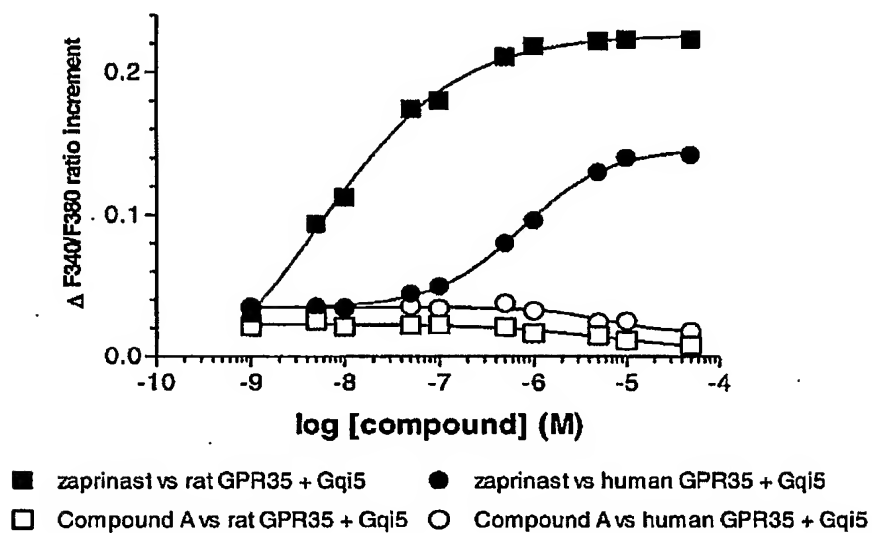
**Figure 4. Coupling of GPR35 with G proteins**



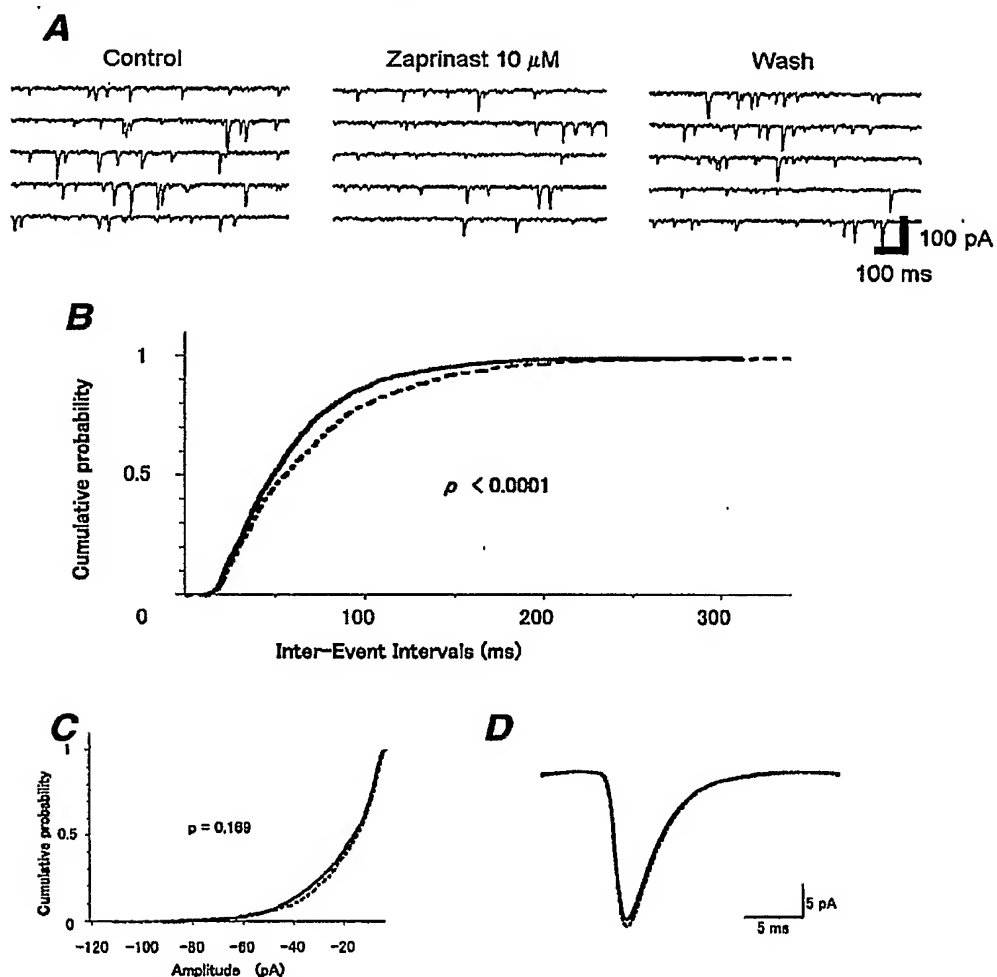
**Figure 5. Effect of zaprinast on rat GPR35**



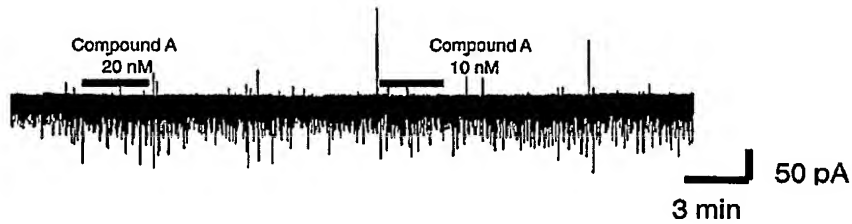
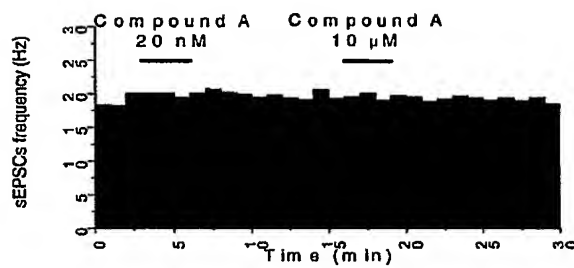
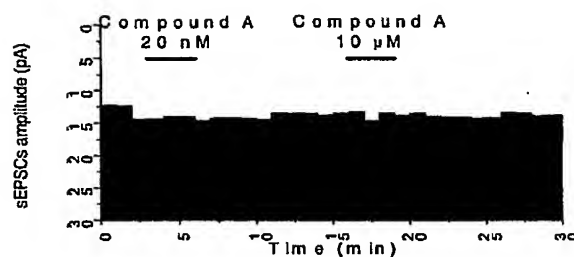
**Figure 6. Experiments to confirm mechanism**



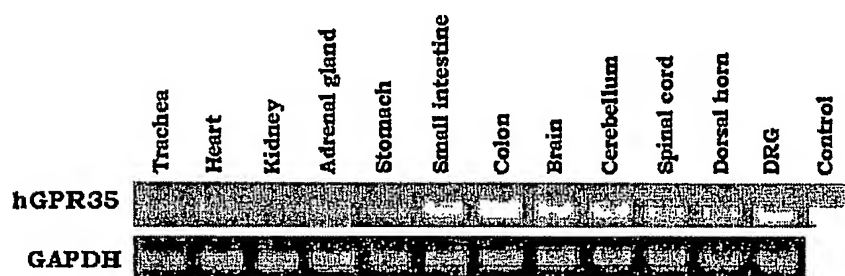
## Figure 7. Effects of zaprinast on mEPSCs



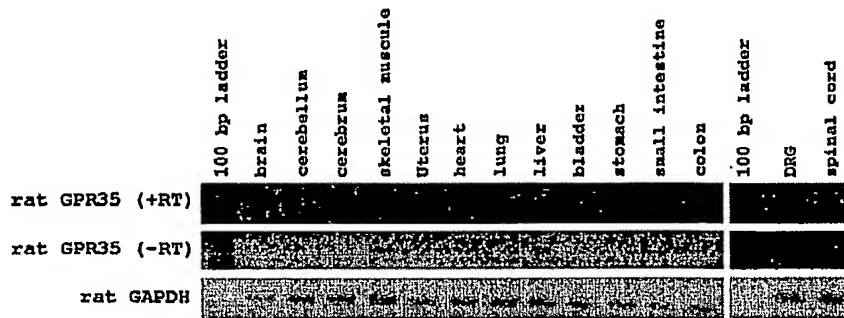
(A) Five consecutive traces of mEPSCs before the action (left), during the action of zaprinast (10  $\mu$ M) (middle; 1min after the beginning of zaprinast application), and after wash out of zaprinast (right). (B, C) Cumulative distribution of inter-event interval (B) and amplitude (C) of mEPSC before (continuous line) and during (dotted line) the action of zaprinast, which were obtained by analyzing 1033 and 879 mEPSC events (which occurred in 1min). (D) The kinetics of mEPSCs before (continuous line) and during (dotted line) the action of zaprinast. Data in A, B, C and D were obtained from the same neuron.

**Figure 8. Effects of Compound A on mEPSCs****A****B****C**

(A) Continuous chart recording of mEPSCs under the action of Compound A (20nM and 10  $\mu$  M) superfused for 3min. (B) Time courses of the frequency (left) and amplitude (right) of mEPSC from the neuron shown in A.

**Figure 9. Tissue distribution of human GPR35**

BEST AVAILABLE COPY.

**Figure 10. Tissue distribution of rat GPR35**

## PC32099\_GPR35(Sequence)

## SEQUENCE LISTING

&lt;110&gt; Pfizer Inc.; Pfizer Japan Inc.(for Japan)

&lt;120&gt; GPR35

&lt;130&gt; PC32099

&lt;160&gt; 23

&lt;170&gt; PatentIn version 3.1

&lt;210&gt; 1

&lt;211&gt; 921

&lt;212&gt; DNA

&lt;213&gt; rat

&lt;400&gt; 1

atgaacaata caaattglag catcctcccg tggcctgctg cagtcaacca catctcacc 60  
 atctactgg tcttgtgctg ggtgctgggc ctgctgtca atggcctggc actctgggta 120  
 ttctgctatc gcatgcacca gtggacggag acccgagtct atatgacaa cctggctgtg 180  
 gctgacgtct gctgctctg ctcttgcca ttctgtctgt actccctgaa atacagtact 240  
 tcggacacac ccatctgcca gctctcacag ggcatctacc tggtaacag gtacatgagc 300  
 ataagcttgg tcaccgccat tgctgtggac cgctatgtgg cagtgcggca tcccctgcgt 360  
 gcccgtagag tgcggcccc acggcaggct ggagcagtgt gtgtggccct ctgggtgata 420  
 gtgtgacct ccttggtact gcgctggcgc ctggggatac aggagggtgg ctctgcttc 480  
 agcagccaaa atcgtlcaa ctccagcacc actgccttct cgctgctggg attctacctg 540  
 ccgctggcca tagtggctt ctgctcttg caggttgtga ctgctggc ccgaaggcca 600  
 gccactgacg tggagcaggt ggaggccact cagaaggcca cccgcatggt ctgggccaac 660  
 ttggccgtgt ttatcatctg ctctctgccc ctgcattga tctgacagt gcaggtctcc 720  
 ctgaacctcc acacctgccc tggccgaaac atctcagcc gtgcctgac aatcacagcc 780  
 aagctctcag acatcaactg ctgctggat gccatctgt actactacat ggccaaagag 840  
 ttccaggatg cgtccttgcg ggccacagcc tctagcacac ccacaagag ccaagatact 900  
 cagagcctga gctcaccta g 921

&lt;210&gt; 2

&lt;211&gt; 306

&lt;212&gt; PRT

&lt;213&gt; rat

&lt;400&gt; 2

Met Asn Asn Thr Asn Cys Ser Ile Leu Pro Trp Pro Ala Ala Val Asn  
 1 5 10 15

His Ile Phe Thr Ile Tyr Leu Val Leu Leu Leu Val Leu Gly Leu Leu  
 20 25 30

Leu Asn Gly Leu Ala Leu Trp Val Phe Cys Tyr Arg Met His Gln Trp  
 35 40 45

□□□(1)

## PC32099\_GPR35(Sequence)

Thr Glu Thr Arg Val Tyr Met Thr Asn Leu Ala Val Ala Asp Val Cys  
50 55 60

Leu Leu Cys Ser Leu Pro Phe Val Leu Tyr Ser Leu Lys Tyr Ser Thr  
65 70 75 80

Ser Asp Thr Pro Ile Cys Gln Leu Ser Gln Gly Ile Tyr Leu Val Asn  
85 90 95

Arg Tyr Met Ser Ile Ser Leu Val Thr Ala Ile Ala Val Asp Arg Tyr  
100 105 110

Val Ala Val Arg His Pro Leu Arg Ala Arg Glu Leu Arg Ser Pro Arg  
115 120 125

Gln Ala Gly Ala Val Cys Val Ala Leu Trp Val Ile Val Val Thr Ser  
130 135 140

Leu Val Leu Arg Trp Arg Leu Gly Ile Gln Glu Gly Gly Phe Cys Phe  
145 150 155 160

Ser Ser Gln Asn Arg Tyr Asn Phe Ser Thr Thr Ala Phe Ser Leu Leu  
165 170 175

Gly Phe Tyr Leu Pro Leu Ala Ile Val Val Phe Cys Ser Leu Gln Val  
180 185 190

Val Thr Ala Leu Ala Arg Arg Pro Ala Thr Asp Val Glu Gln Val Glu  
195 200 205

Ala Thr Gln Lys Ala Thr Arg Met Val Trp Ala Asn Leu Ala Val Phe  
210 215 220

Ile Ile Cys Phe Leu Pro Leu His Leu Ile Leu Thr Val Gln Val Ser  
225 230 235 240

Leu Asn Leu His Thr Cys Ala Ala Arg Asn Ile Phe Ser Arg Ala Leu  
245 250 255

Thr Ile Thr Ala Lys Leu Ser Asp Ile Asn Cys Cys Leu Asp Ala Ile  
260 265 270

Cys Tyr Tyr Tyr Met Ala Lys Glu Phe Gln Asp Ala Ser Leu Arg Ala  
275 280 285

Thr Ala Ser Ser Thr Pro His Lys Ser Gln Asp Thr Gln Ser Leu Ser  
290 295 300

Leu Thr  
305

□□□(2)

PC32099\_GPR35(Sequence)

<210> 3  
<211> 930  
<212> DNA  
<213> human

<400> 3  
atgaatggca cctacaacac ctgtggctcc agcgacctca cctggccccc agcgaatcaag 60  
ctgggcttct acgcctactt gggcgctctg ctgggtgtag gcctgctgct caacagcctg 120  
gcgctctggg tgtctgctg ccgcatgcag cagtggacgg agaccgcgat ctacatgacc 180  
aacctggcgg tggccgacct ctgcctgctg tgcacctgc ccttcgtgct gcactccctg 240  
cgagacacct cagacacgcc gctgtgccag ctctcccagg gcattctacct gaccaacagg 300  
tacatgagca tcagcctggt catggccatc gccgtggacc gctatgtggc cgtgcggcac 360  
ccgctgcgtg cccgcggggt gcggctcccc aggcaggctg cggccgtgtg cgcggctctc 420  
tgggtgctgg tcatcggtc cctggtggct cgctggctcc tggggattca ggagggcggc 480  
ttctgttca ggagcaccgc gcacaattc aactccatgg cgttcccgt gctgggattc 540  
tacctgcccc tggccgtggt ggtctctgc tccctgaagg tgggtactgc cctggcccag 600  
aggccacca cgcagctggg gcaggcagag gccaccgcga aggtgcccg catggtctgg 660  
gccaacctcc tgggtgtcgt ggtctgctc ctgccctgc acgtggggct gacagtgcgc 720  
ctgcagtggt gctggaacgc ctgtgccctc ctggagacga tccgtcgcgc cctgtacata 780  
accagcaagc tctcagatgc caactgctgc ctggacgcca tctgtacta ctacatggcc 840  
aaggagtcc aggaggcgtc tgcactggcc gtggctccca gtgctaaggc ccacaaaagc 900  
caggactctc tgtgcgtgac cctgcctaa 930

<210> 4  
<211> 309  
<212> PRT  
<213> human

<400> 4

Met Asn Gly Thr Tyr Asn Thr Cys Gly Ser Ser Asp Leu Thr Trp Pro  
1 5 10 15

Pro Ala Ile Lys Leu Gly Phe Tyr Ala Tyr Leu Gly Val Leu Leu Val  
20 25 30

Leu Gly Leu Leu Leu Asn Ser Leu Ala Leu Trp Val Phe Cys Cys Arg  
35 40 45

Met Gln Gln Trp Thr Glu Thr Arg Ile Tyr Met Thr Asn Leu Ala Val  
50 55 60

Ala Asp Leu Cys Leu Leu Cys Thr Leu Pro Phe Val Leu His Ser Leu  
65 70 75 80

□□□(3)

## PC32099\_GPR35(Sequence)

Arg Asp Thr Ser Asp Thr Pro Leu Cys Gln Leu Ser Gln Gly Ile Tyr  
 85 90 95

Leu Thr Asn Arg Tyr Met Ser Ile Ser Leu Val Met Ala Ile Ala Val  
 100 105 110

Asp Arg Tyr Val Ala Val Arg His Pro Leu Arg Ala Arg Gly Leu Arg  
 115 120 125

Ser Pro Arg Gln Ala Ala Val Cys Ala Val Leu Trp Val Leu Val  
 130 135 140

Ile Gly Ser Leu Val Ala Arg Trp Leu Leu Gly Ile Gln Glu Gly Gly  
 145 150 155 160

Phe Cys Phe Arg Ser Thr Arg His Asn Phe Asn Ser Met Ala Phe Pro  
 165 170 175

Leu Leu Gly Phe Tyr Leu Pro Leu Ala Val Val Val Phe Cys Ser Leu  
 180 185 190

Lys Val Val Thr Ala Leu Ala Gln Arg Pro Pro Thr Asp Val Gly Gln  
 195 200 205

Ala Glu Ala Thr Arg Lys Ala Ala Arg Met Val Trp Ala Asn Leu Leu  
 210 215 220

Val Phe Val Val Cys Phe Leu Pro Leu His Val Gly Leu Thr Val Arg  
 225 230 235 240

Leu Ala Val Gly Trp Asn Ala Cys Ala Leu Leu Glu Thr Ile Arg Arg  
 245 250 255

Ala Leu Tyr Ile Thr Ser Lys Leu Ser Asp Ala Asn Cys Cys Leu Asp  
 260 265 270

Ala Ile Cys Tyr Tyr Tyr Met Ala Lys Glu Phe Gln Glu Ala Ser Ala  
 275 280 285

Leu Ala Val Ala Pro Ser Ala Lys Ala His Lys Ser Gln Asp Ser Leu  
 290 295 300

Cys Val Thr Leu Ala  
 305

<210> 5

<211> 924

<212> DNA

<213> mouse

<400> 5

atgaatagta caacctgtaa cagcaccctc acgtggcctg cttcgtcaa caactcttc 60

□□□(4)

## PC32099\_GPR35(Sequence)

atcatctact cagccttgct gctgggtctg ggctgtctgc tcaacagcgt ggcactctgg 120  
 gtattctgct atcgcatgca ccagtggaaga gagaccgca tctatatgac caacctggct 180  
 gtggccgacc tctgcctgct ctgtctcttg ccatttgctg tgtactccct gaaatatagt 240  
 tcttcagaca caccgctctg ccagctctca cagggcatct acctggccaa cagatcatg 300  
 agcataagcc tggctactgc cattgctgtg gaccgctatg tggcagtgcg gcatccccctg 360  
 cgtgcgcgtg agctgcgggc cccgagacag gctgcagcag tgtgtgtggc ccttgggtg 420  
 atagtgtgta cctccctggg agtgcgctgg cgctgtggga tgcaggaggg tggcttctgc 480  
 ttcagcagcc aaaccggcg caatttcagc accactgcct tctactgct gggattctac 540  
 ctgccgctgg ccatcgtggt ctctgctct ttgcaggtag tgactgtgct atcgagaagg 600  
 ccagccgctg atgtggggca ggcagaggcc acccaaaagg ccaccacat ggtctgggcc 660  
 aacttgctg tggttgcat ctgtctctg ccctgcatg tggctctgac cgtgcaggtc 720  
 tccctgaacc tcaatacctg tgctgccga gacacctca gccgtgccct gtccatcaca 780  
 ggtaaacctc cagacaccaa ctgtgcctg gatgccatct gtactacta catggccaga 840  
 gatttcagg aagcgtocaa gccagccacg tctccaaca caccacaaa gagccaagat 900  
 tccagatcc tgagcctcac ctag 924

<210> 6  
 <211> 307  
 <212> PRT  
 <213> mouse

<400> 6

Met Asn Ser Thr Thr Cys Asn Ser Thr Leu Thr Trp Pro Ala Ser Val  
 1 5 10 15

Asn Asn Phe Phe Ile Ile Tyr Ser Ala Leu Leu Leu Val Leu Gly Leu  
 20 25 30

Leu Leu Asn Ser Val Ala Leu Trp Val Phe Cys Tyr Arg Met His Gln  
 35 40 45

Trp Thr Glu Thr Arg Ile Tyr Met Thr Asn Leu Ala Val Ala Asp Leu  
 50 55 60

Cys Leu Leu Cys Ser Leu Pro Phe Val Leu Tyr Ser Leu Lys Tyr Ser  
 65 70 75 80

Ser Ser Asp Thr Pro Val Cys Gln Leu Ser Gln Gly Ile Tyr Leu Ala  
 85 90 95

Asn Arg Tyr Met Ser Ile Ser Leu Val Thr Ala Ile Ala Val Asp Arg  
 100 105 110

Tyr Val Ala Val Arg His Pro Leu Arg Ala Arg Glu Leu Arg Ser Pro  
 115 120 125

□□□(5)

## PC32099\_GPR35(Sequence)

Arg Gln Ala Ala Val Cys Val Ala Leu Trp Val Ile Val Val Thr  
 130 135 140

Ser Leu Val Val Arg Trp Arg Leu Gly Met Gln Glu Gly Gly Phe Cys  
 145 150 155 160

Phe Ser Ser Gln Thr Arg Arg Asn Phe Ser Thr Thr Ala Phe Ser Leu  
 165 170 175

Leu Gly Phe Tyr Leu Pro Leu Ala Ile Val Val Phe Cys Ser Leu Gln  
 180 185 190

Val Val Thr Val Leu Ser Arg Arg Pro Ala Ala Asp Val Gly Gln Ala  
 195 200 205

Glu Ala Thr Gln Lys Ala Thr His Met Val Trp Ala Asn Leu Ala Val  
 210 215 220

Phe Val Ile Cys Phe Leu Pro Leu His Val Val Leu Thr Val Gln Val  
 225 230 235 240

Ser Leu Asn Leu Asn Thr Cys Ala Ala Arg Asp Thr Phe Ser Arg Ala  
 245 250 255

Leu Ser Ile Thr Gly Lys Leu Ser Asp Thr Asn Cys Cys Leu Asp Ala  
 260 265 270

Ile Cys Tyr Tyr Tyr Met Ala Arg Glu Phe Gln Glu Ala Ser Lys Pro  
 275 280 285

Ala Thr Ser Ser Asn Thr Pro His Lys Ser Gln Asp Ser Gln Ile Leu  
 290 295 300

Ser Leu Thr  
 305

<210> 7  
 <211> 61  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> primer

<400> 7  
 ccggaattcg ccaccatgga ttacaaggat gacgacgata agaatggcac ctacaacacc 60

t 61

<210> 8  
 <211> 30  
 <212> DNA  
 <213> Artificial Sequence

□□□(6)

## PC32099\_GPR35(Sequence)

<220>  
<223> primer

<400> 8  
tcgtctagaa ttaggcgagg gtcacgcaca 30

<210> 9  
<211> 36  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> primer

<400> 9  
ccggaattcg ccaccatgaa tggcacctac aacacc 36

<210> 10  
<211> 40  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> primer

<400> 10  
ccccgaattc gccaccatga atagtacaac ctgtaacagc 40

<210> 11  
<211> 34  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> primer

<400> 11  
ccccctcgag ctaggtagg ctcaggatct ggga 34

<210> 12  
<211> 23  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> primer

<400> 12  
tcgctcagat gagccctagg acc 23

<210> 13  
<211> 26  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> primer

<400> 13  
cacaggttcc tctggccctt ggcattg 26

□□□(7)

## PC32099\_GPR35(Sequence)

<210> 14  
 <211> 36  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> primer

<400> 14  
 cccogaattc gccaccatga acaatacaaa ttgtag 36

<210> 15  
 <211> 30  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> primer

<400> 15  
 cccctcgag ctaggtagg ctcaggctct 30

<210> 16  
 <211> 25  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> primer

<400> 16  
 cactccctgc gagacacctc agaca 25

<210> 17  
 <211> 25  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> primer

<400> 17  
 tagtagcaga tggcgtccag gcagc 25

<210> 18  
 <211> 17  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> primer

<400> -18  
 tgcaccacca actgctt 17

<210> 19  
 <211> 17  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> primer

□□□(8)

## PC32099\_GPR35(Sequence)

<400> 19  
tactccttgg aggccat

17

<210> 20  
<211> 24  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> primer

<400> 20  
aaattgtagc atctcccggt ggcc

24

<210> 21  
<211> 24  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> primer

<400> 21  
tatctggct ctgtggggt gtgc

24

<210> 22  
<211> 24  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> primer

<400> 22  
gtcttcacca ccatggagaa ggct

24

<210> 23  
<211> 25  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> primer

<400> 23  
gtgatggcat ggactgtggt catga

25